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**The role of apoptosis and expression of bcl2 and CD40
in squamous cell carcinoma of the lung.**

by

Dr. Monimoy Ghosh M.B.B.S., M.R.C.P.

A thesis presented to the University of Warwick for the degree of M.D.

Submitted December 1997

For Daddy and Ma

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Declaration

All work reported in this thesis was performed by the author (except where stated) in the laboratory of Dr. Alan Morris at the Biological Sciences Department, University of Warwick and the laboratory of Dr. John Crocker at Birmingham Heartlands Hospital.

Use was made of information available from previous investigations by Boldy and colleagues on the same set of cases of lung cancer that we examined. They had determined histological grade of differentiation, AgNOR counts and DNA ploidy from paraffin sections of lung cancer and tried to correlate it with clinical outcome. These findings had been published in Thorax (Boldy *et al.*, 1991).

Work described in this thesis has not been submitted for a degree at any other institution.

List of Abbreviations

ABC	avidin biotin complex	FCS	foetal calf serum
AC	adenocarcinoma	FITC	fluorescein isothiocyanate
AgNOR	Ag (silver) nucleolar organizer region	GRP	gastrin releasing peptide
AHH	aryl hydrocarbon hydroxylase	GTP	guanosine triphosphate
AI	apoptotic index	GM-CSF	granulocyte macrophage colony stimulating factor
AIDS	acquired immune deficiency syndrome	HBV	hepatitis B virus
ALS	amyotrophic lateral sclerosis	HD	Hodgkin's disease
APC	antigen presenting cell	H&E	haematoxylin and eosin
ASP	apoptosis specific protein	HIV	human immunodeficiency virus
ATP	adenosine triphosphate	ICE	interleukin 1- β converting enzyme
BCC	basal cell carcinoma	IFN	interferon
bcl2	bcl2 gene	IGF	insulin like growth factor
Bcl2	Bcl2 protein	IL(-1)	interleukin(-1)
BI	Bcl2 staining index	IP3	inositol triphosphate
BL	Burkitt's lymphoma	ISCC	invasive squamous cell carcinoma
BP	benzo (a) pyrene	ISEL	in situ end labelling
CD	cluster designation	LC	large cell carcinoma
CIS	carcinoma <i>in situ</i>	LOH	loss of heterozygosity
CI	CD40 staining index	MM	malignant melanoma
CIN	cervical intraepithelial neoplasia	MMP	matrix metalloproteinase
CML	chronic myeloid leukaemia	mRNA	messenger ribonucleic acid
CTL	cytotoxic T-lymphocyte	NE	neuroendocrine
DAB	3,3 - diaminobenzidine tetrahydrochloride	NHL	non-hodgkin's lymphoma
DAG	diacyl glycerol	NK	natural killer
DMSO	dimethyl sulfoxide	NSCLC	non-small cell lung carcinoma
DNA	deoxyribonucleic acid	ODC	ornithine decarboxylase
DNase	deoxyribonuclease	p53	p53 gene
ds	double stranded	P53	P53 protein
EBV	Epstein-Barr virus	PAH	polycyclic aromatic hydrocarbon
ECM	extra cellular matrix	PARP	poly (ADP ribose) polymerase
EDTA	ethylene-diamino-tetra-acetic acid	PBS	phosphate buffered saline
EGF	epidermal growth factor	PCNA	proliferating cell nuclear antigen
ER	endoplasmic reticulum	PDGF	platelet derived growth factor
FACS	fluorescence activated cell sorter		

PE	pleural effusion	TIMP	tissue inhibitors of
PI	propidium iodide		metalloproteinases
PIP2	phosphatidyl inositol	TNF	tumour necrosis factor
	biphosphate	TNFR	tumour necrosis factor
PKC	protein kinase C		receptor
PLC	phospholipase C	TRAF	TNF receptor associated
PS	phosphatidyl serine		factor
RNA	ribonucleic acid	TUNEL	terminal
RNAse	ribonuclease		deoxynucleotidyl
ROS	reactive oxygen species		transferase (TdT)
RPMI	Roswell park memorial		mediated biotin-dUTP
	institute		nick end labelling of
SCC	squamous cell carcinoma		DNA
SCLC	small cell lung carcinoma	UNPC	undifferentiated naso-
SLE	systemic lupus		pharyngeal carcinoma
	erythematosus	UV	ultraviolet
TGF	transforming growth		
	factor		

Summary

In spite of extensive research there is little information about apoptosis or programmed cell death in the genesis and progression of cancers of the lung. In our project we have investigated the role of apoptosis and two of the genes controlling apoptosis (bcl2 and CD40) in squamous cell carcinoma of the lung. Also we have tried to formulate an accurate way of measuring apoptotic rate in tumour specimens.

We counted apoptotic cells in Haematoxylin and Eosin stained histological sections of squamous cell carcinoma of the lung. The apoptotic indices we obtained were very reliable showing remarkable reproducibility and strong correlation with apoptosis measured by monoclonal antibody to apoptosis specific protein (ASP). In our series apoptotic index did not correlate with survival, disease stage, differentiation, AgNOR or DNA ploidy.

Histological sections were stained with monoclonal antibodies to Bcl2 and CD40. In all, 32% of squamous cell carcinoma of lung were Bcl2 positive (i.e. more than 50% of the tumour cells contained Bcl2 protein) and 22% were positive for CD40. The expression of Bcl2 correlated positively with the apoptotic indices. Patients with Bcl2 positive squamous cell lung cancers survived significantly longer although Bcl2 expression did not correlate with any marker of disease severity e.g. stage, grade, AgNOR or DNA ploidy.

CD40 expression in squamous cell lung cancer had no effect on apoptosis, survival or any of the other previously mentioned markers of disease severity. The expression of CD40 in our series showed a tendency to correlate inversely with the expression of Bcl2.

We devised a way to measure apoptotic rate in primary cultures of tumour cells by serial estimation of sub-diploid fractions in cell suspensions double stained with PI and BerEP4-FITC. We believe that the apoptotic rate measured in this fashion is biologically more relevant, and therefore could be more useful in predicting prognosis, than apoptosis measured from histological sections.

CHAPTER 1

INTRODUCTION

Preface

Inspite of rapid advances in medical technology cancer still remains a scourge of humanity, inspiring awe and fear both in the lay person and among medical professionals. An incomplete understanding of the biology of cancer is felt to be one of the main impediments to devising successful ways of combating it. Being a chest physician my interest is in the area of lung cancer and I have chosen to look at one particular aspect of its biology - namely the role of apoptosis and the genes controlling it in the development and progression of lung cancer.

This introductory chapter will provide the background for our experimental work and has been laid out in four sections. The first section is on lung cancer where we shall briefly describe the common types of pulmonary neoplasms followed by a discussion of the various aetiological factors involved. Next we shall describe the steps involved in the transformation of normal bronchial epithelium to malignancy with particular reference to the role of apoptosis and apoptosis controlling genes in this multi-step process.

Section two is on apoptosis. The morphological features and biochemical mechanisms of this newly discovered physiological process are described here in some detail. Following this, the importance of this process in various pathological conditions and in particular its role in cancer is discussed in depth.

The final two sections deal with two protein molecules which regulate apoptosis in cells - namely Bcl2 and CD40. In each of these sections a description of the structure and the distribution of the protein is followed by a discussion of its normal role in various physiological processes and altered role in cancer.

1.1 Lung Cancer

1.1.1 Introduction

At the beginning of the twentieth century lung cancer was a rare, essentially reportable condition but it has now become the commonest fatal neoplastic disease in the world. In the United States alone around 150,000 new cases appear annually. In the United Kingdom it is estimated that every year there are 300 deaths due to lung cancer per 100,00 male population (Office of Population Censuses and Surveys, 1987). It is the most common cancer in British men and in women takes second place only to cancer of the breast.

The predominant cause of this epidemic is well known. For more than four decades, scientists and physicians have recognised the inextricable link between cigarette smoking and lung cancer. In spite of this well recognised relationship, only in recent years have men and women in parts of the Western world started to curtail their use of tobacco. Sadly, a similar trend has not been noticed in many other parts of the world. Worse yet, increased tobacco use is the norm for most underdeveloped countries and even some industrialised nations. Thus, lung cancer is and will remain a significant health problem throughout the world for many years to come.

There are no effective ways of treating this disease and it carries a very poor prognosis. The median survival is 12 months and only 13% of patients are still alive at 5 years after diagnosis. Introduction of new modalities of treatment (e.g. adjuvant chemotherapy following surgery in NSCLC) has not appreciably dented these dismal figures. It is widely felt that a deeper understanding of the biology of this dreadful disease might lead to new avenues of managing it more effectively. Understanding the pathogenesis of lung cancer should help in devising new prevention strategies, strategies for early diagnosis (maybe even at a pre-malignant stage) and strategies for new innovative means of treating this cancer.

Recently much attention has been focused on one particular aspect of lung cancer biology namely the role of apoptosis and apoptosis controlling genes in the genesis and development of pulmonary neoplasms. Although extensive research has been carried out, a lot still remains to be known about this fundamental physiological process and its relative importance amongst myriad other pathological changes seen in lung cancer.

1.1.2 Clinical Pathology

Lung cancer is not a single entity but a generic term applied to a heterogeneous group of malignancies (Appendix A). Most cancers of the lung are epithelial tumours, or carcinomas. In contrast to other common cancers, such as cancers of the colon, breast and prostate, which are mostly adenocarcinomas, there are four main histological types of lung cancer, each with distinct biological characteristics. They are squamous cell carcinoma (50%), adenocarcinoma (15%), large cell carcinoma (10%) and small cell carcinoma (25%). Clinically and therapeutically, lung cancer is divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) which includes all the other carcinomas.

Below is a brief account of the four common types of lung cancer followed by a short discussion on malignant pleural effusions which is a common clinical manifestation of cancers of the lung and which formed part of our investigations.

1.1.2.1 Squamous cell carcinoma

This is the most common type of lung cancer. It usually affects males and is closely correlated with a smoking history. Squamous cell carcinomas usually arise in the large central bronchi. The epithelium in the normal tracheobronchial tree does not contain squamous cells but over the years an area of altered bronchial mucosa undergoes progressive changes which include hyperplasia, squamous metaplasia, dysplasia and carcinoma *in situ* eventually leading to frank carcinoma. The histological features of squamous cell carcinoma are distinctive (see Fig. 1.1). Tumour cells which are arranged in nests or pseudoductal pattern, are stratified and show intercellular bridge formation and

visible keratinization. Extensive accumulation of keratin may lead to formation of keratin pearls. While squamous cell carcinoma may grow very rapidly, they often remain confined within the thoracic cavity and the incidence of extra-thoracic metastases (haematogeneous and lymphatic) is somewhat less than in the other histologic types (Seaton *et al.*, 1989).

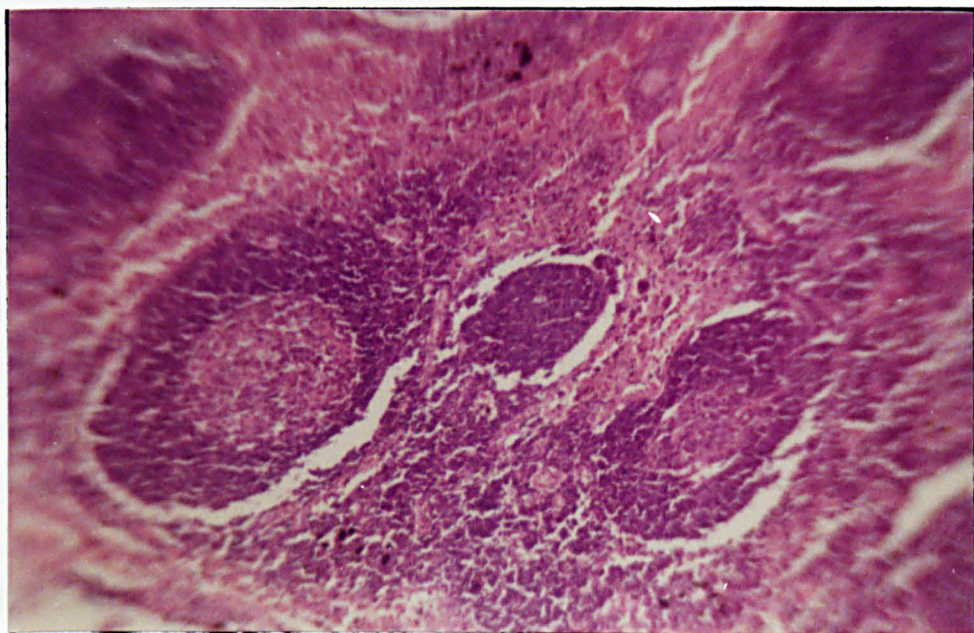


Fig. 1.1 Histological section of squamous cell carcinoma of lung ($\times 250$) stained with H&E showing tumour cells arranged in nests.

The patient is usually a middle aged male smoker who presents with any or all of the following symptoms—breathlessness, cough, haemoptysis and chest pain, or a symptom referable to a site of distant metastasis. Occasionally the disease is asymptomatic and first comes to light on a ‘routine’ chest X-ray. The diagnosis is established by chest X-ray, CT scan and bronchoscopic biopsy or percutaneous aspiration cytology. All NSCLCs (including squamous cell carcinoma) are treated along identical lines although the response varies (see below) (Minna, 1991).

Surgery is the major curative modality for patients without demonstrable metastatic disease. Of the surgically treated patients the majority will relapse with local recurrence, extra-thoracic metastases or a second primary tumour. To delay or prevent this from happening, adjuvant chemotherapy following operation has been recommended, but the

results have not been uniformly favourable (Tonato, 1996). Unfortunately more than two thirds of NSCLC patients are unresectable at presentation. These patients are treated with a combination of chemotherapy and radiotherapy.

Patients vary in their response to treatment. In general squamous cell carcinomas fare much better than adenocarcinomas or large cell carcinomas, but even among the same histological type and the same clinical stage there is often variation in response. Several factors have been shown to influence outcome after surgical treatment: immunological markers such as PCNA, Ki-67, ras and p53, tumour DNA content (ploidy), perioperative blood transfusion, and presence of certain blood group antigens (reviewed by Mountain, 1995). Response to chemotherapy is influenced by a different set of factors one of which is the presence of neuroendocrine (NE) features (see SCLC below). Almost all SCLCs and only about 10-15% of NSCLCs demonstrate features of NE differentiation. The subgroup of NSCLCs which have NE features respond more readily to chemotherapy than do tumours without these features (Linnoila *et al.*, 1989). It is important to investigate other biological features of tumours which may influence response to surgery, chemotherapy or radiotherapy. The results of such efforts could help select the ideal treatment for a particular patient and thus dramatically alter how NSCLC is managed.

1.1.2.2 Adenocarcinoma

Adenocarcinoma is the commonest type of lung cancer in women and non-smokers, even though most adenocarcinoma patients do smoke. The incidence of this cancer has increased over the last decade and in the United States it has now become the most common form of lung cancer (Devesa *et al.*, 1991). Adenocarcinomas usually arise at the periphery of the lung often at the vicinity of old scars e.g. old TB, pulmonary infarcts (see Section 1.1.3.5). These tumours originate from cells normally found distal to the terminal bronchioles i.e. mucin secreting bronchiolar cells, Clara cells or Type II (granular) pneumocytes. They are unrelated to the bronchi other than by spread, except in a few cases where the tumours are truly bronchogenic, arising from mucous glands in the proximal bronchi. Microscopically the tumour consists of gland-like structures which often produce mucin. Adenocarcinomas grow more slowly than squamous cell carcinomas but

they tend to penetrate pleural lymphatics and metastasise to distant sites even before they produce any symptoms in the chest.

Bronchiolo-alveolar carcinomas are an important subgroup of adenocarcinomas which arise from terminal bronchioles and alveolar walls. They comprise about 5% of lung cancers. The tumour almost always occurs in the peripheral portions of the lung either as a single nodule or, more often, as multiple diffuse nodules that sometimes coalesce to produce a pneumonia-like consolidation. Microscopically the tumour is characterised by distinctive tall columnar epithelial cells that spread along the walls of alveoli and bronchioles using these structures as supporting stroma (Edwards, 1984).

1.1.2.3 Large cell carcinoma

This is a heterogeneous group that has been formed in a negative way to accommodate those 'left over' tumours that do not show the distinguishing features of squamous cell, small cell or adenocarcinoma (Patton *et al.*, 1951). They tend to be anaplastic showing no signs of maturation on light microscopy and having large, less well differentiated polygonal, spindle-shaped or oval cells with abundant cytoplasm. Many of these tumours represent undifferentiated forms of adenocarcinoma or squamous cell carcinoma. These tumours arise towards the lung periphery and are large and bulky and often cavitate. Surgical treatment offers a significant chance of cure in large cell carcinoma as in the other NSCLCs such as squamous cell carcinoma and adenocarcinoma.

1.1.2.4 Small cell carcinoma

This highly malignant tumour has a distinctive cell type. The epithelial cells are generally small, have little cytoplasm (high nuclear:cytoplasmic ratio) and are round and oval, and, occasionally lymphocyte-like (although they are about twice the size of a lymphocyte). In 1926 WG Barnard gave the first detailed histologic description of this tumour and likened these small oval tumour cells to that of oat grains (Barnard, 1926). The cells grow in clusters that exhibit neither glandular nor squamous organisation. These cells are thought to arise from the neuroendocrine (NE) cells that are normally present in bronchial epithelium. Like the NE cells oat cells have been shown to contain neurosecretory

granules and some of the tumours are able to secrete polypeptide hormones. Such “ectopic” hormone production is a characteristic feature of oat cell carcinomas.

Small cell carcinomas have a strong relationship to cigarette smoking, and only about 1% occurs in non-smokers. The tumours usually develop proximally in the large bronchi, grow rapidly and metastasise early and widely. At presentation over two thirds of patients have evidence of metastatic disease in the liver, central nervous system and bone (Cohen and Matthews, 1978). Surgery is therefore not an option. In contrast to all other forms of lung cancer small cell carcinoma is very sensitive to both chemotherapy and radiotherapy.

1.1.2.5 Malignant pleural effusions

An abnormal accumulation of fluid in the pleura (a closed serosal space separating the lungs from the chest wall) is a common manifestation of malignancy in the lungs but it occurs in other non-malignant conditions as well.

The patient usually presents with shortness of breath, dry cough and a dull ache over the chest—although if the fluid accumulates slowly over months even a large effusion may produce few symptoms. The classical signs on examination are diminished breath sounds, dullness on percussion and mediastinal shift to the opposite side. On chest X-ray there is usually an opacity in the lower zone with an upward concave level. The disease causing the effusion is determined by aspirating a portion of the fluid for biochemical, cytological and bacteriological tests. Often, histological examination of a pleural biopsy is needed as well.

Pleural effusions may or may not be associated with disease of the pleura. In general, effusions due to pleural disease more nearly resemble plasma (exudates), while those occurring with a normal pleura are ultrafiltrates of the plasma (transudates). Of the latter the commonest cause is congestive cardiac failure which often leads to bilateral pleural effusions due to alterations in hydrostatic pressure across a normal pleura. Hypoalbuminaemia, as occurs in nephrotic syndrome and cirrhosis, also leads to increased formation and decreased reabsorption of pleural liquid on the basis of decreased intravascular oncotic pressures.

Various non-malignant diseases can cause pleural effusions. Bacterial pneumonia is associated with pleural effusion in about 40% of cases. (Light *et al.*, 1980) Tuberculosis is the commonest cause in certain parts of the world. Other uncommon causes include connective tissue disorders like rheumatoid disease, pulmonary infarction, pancreatitis, uraemia and sarcoidosis.

Malignant involvement of the pleura may be primary or secondary to tumours elsewhere. Primary pleural tumour is almost always mesothelioma which is strongly associated with exposure to asbestos and carries a poor prognosis. It is often difficult to distinguish this tumour cytologically and histologically from secondary adenocarcinomas in the pleura. The BerEP4 monoclonal antibody has been found to be useful in making this distinction (Sheibani *et al.*, 1991; Dejmek and Hjerpe, 1994; Hartmann and Schutze, 1994. Also see Section 3.1.5 a).

Bronchogenic carcinoma is the commonest malignancy causing pleural effusion and may do so by direct extension to the pleural surface, obstruction to lymphatic drainage (secondary to mediastinal spread), or by pleural inflammation secondary to pneumonia behind an obstructed bronchus. Almost any other malignant neoplasm may occasionally metastasise to the pleura, common sources being breast, stomach and ovary (Chernow and Sahn, 1977). Lymphoma may directly involve the pleura or obstruct lymphatic drainage leading to a pleural effusion.

Malignant effusions are characteristically heavily blood-stained and protein rich and tend to reaccumulate quickly within days of aspiration. Although the presence of effusion indicates a poor prognosis, only those patients with demonstration of tumour cells in the pleural fluid or on closed pleural biopsy samples are considered unresectable (Mountain, 1985). Other patients with effusion are considered to have resectable lesions, despite their poor prognosis.

That the malignant cells in effusions survive and even thrive in an environment where they are deprived of some of the usual survival signals (mediated by cell-cell and cell-ECM contact; see Section 1.2.3.2) indicate that they may represent a specially selected group of cells. One aspect that may differentiate them from the cells in the mother tumour might be an extra resistance to apoptosis.

1.1.3 Aetiological factors in lung cancer

A number of extraneous factors contribute to the development of lung cancer. The foremost amongst these is cigarette smoking which is responsible for the majority of lung cancers. In comparison the other factors play a relatively negligible role but are nevertheless important in that many of them can cooperate with cigarettes and increase many fold the effectiveness of the carcinogens within cigarette smoke.

1.1.3.1 Cigarette smoking

King James I of England expressed his dislike of tobacco smoking forcibly in 1604, describing it as 'loathsome to the eye, hateful to the nose, harmful to the brain, *dangerous to the lungs*, and in the black stinking fume thereof nearest resembling the horrible Stygian smoke of the pit that is bottomless' (Stuart, 1604). These early Royal misgivings have been amply justified and extensive research has shown that cigarette tobacco smoking is by far the world's most common cause of lung cancer. It is estimated that world wide over 90% of lung cancer deaths are attributable to tobacco smoking. The overwhelming evidence against tobacco can be arrayed under the following headings:-

a) epidemiological evidence, b) pathological evidence, c) biochemical evidence, d) molecular genetic evidence and e) animal experiments.

a) Epidemiological evidence

Three hospital-based case control studies published in the early 1950s provided evidence that is now regarded as conclusive in establishing the causal link between cigarette smoking and lung cancer. Two English investigators compared the smoking habits of more than 1300 lung cancer patients in London hospitals to that of a matched group of controls (Doll and Hill, 1952). Two American groups carried out similar studies – all three demonstrated that a significantly greater proportion of the lung cancer patients were smokers (Levin *et al.*, 1950; Wynder and Graham, 1950). Convincing support was provided by further prospective trials. Doll and Peto correlated the smoking habits of over 34,000 British physicians with mortality from lung cancer over a period of 20 years and

found a significant decline both in cigarette smoking and mortality in this group in contrast with the general population in whom neither mortality rates from this disease nor tobacco consumption fell (Doll and Peto, 1976).

It is estimated that cigarette smokers are 8-20 times more likely to develop lung cancer than life-long non-smokers. The extent of the risk correlates with the duration of smoking, the number of cigarettes smoked, the depth of inhalation, the tar content of cigarettes and the presence of filter tips. Pipe and cigar smokers are at a lower risk because less smoke is inhaled. There is no lower limit of exposure below which there is no risk and even 'passive smokers' (those who involuntarily inhale side stream smoke that is emitted into the environment while an active smoker pursues his habit) have an approximately 30% increment in risk. About a quarter of the cases of lung cancer in non-smokers can be attributed to passive smoking (Wald *et al.*, 1986). Cessation of smoking progressively lowers the risk of lung cancer with time, to about two and a half times that of non-smokers by 10-20 years.

It is likely that the incidence of all principal histological types of lung cancer is increased by tobacco smoking but those cell types with the clearest association are squamous cell and small cell carcinoma. There is also epidemiologic evidence to show an association between cigarette smoking and the following cancers in decreasing order of frequency:- lip, tongue, floor of mouth, pharynx, larynx, oesophagus, urinary bladder and pancreas.

b) Pathological evidence

The genesis of lung cancer is a multi-step process and is preceded by a continuum of pathological changes that include hyperplasia, metaplasia, dysplasia and carcinoma *in situ* (See Section 1.1.4.1) Auerbach and colleagues noted a similar spectrum of histological changes in the tracheo-bronchial tree of the majority of smokers while very few non-smokers showed such pre-cancerous changes. The changes were reversible when the subjects stopped smoking. Also, such pre-cancerous changes are often found in the 'normal' epithelium surrounding a tumour (Auerbach *et al.*, 1961; Auerbach *et al.*, 1962).

c) Biochemical evidence

Tobacco smoke contains over 3800 constituents many of which are carcinogens. They include both initiators (polycyclic aromatic hydrocarbons such as benzo[a]pyrene) and promoters such as phenol derivatives. Radioactive elements may also be found (polonium-210, carbon-14, potassium-40) as well as other contaminants such as arsenic, nickel, moulds and additives. Of these the polycyclic aromatic hydrocarbons (PAH) are some of the most potent carcinogens known. They require metabolic activation and can induce tumours in a wide variety of tissues and species. The primary target of such chemical carcinogens is cellular DNA where they induce mutations (Harris, 1983).

d) Molecular genetic evidence

Mutations in oncogenes and tumour suppressor genes play a crucial role in the pathogenesis of lung cancer as discussed in Section 1.1.4. Many of these mutations are brought on by exposure to chemical carcinogens. Each carcinogen causes a characteristic mutation in DNA. For instance benzo(a) pyrene found in cigarette smoke characteristically causes GC to TA transversions which is the most prevalent type of mutation seen in lung cancer in p53 and K-ras genes (Slebos *et al.*, 1991). This last bit of evidence could be considered the proverbial smoking gun irrevocably proving tobacco's guilt.

e) Animal experiments

Repeated application of a condensate of cigarette smoke to the skin of animals produces cutaneous tumours. Notwithstanding the numerous difficulties of simulating human smoking conditions in animal experiments lung carcinomas have been produced in rats in response to tobacco smoke inhalation and in hamsters following the intra-tracheal instillation of respiratory carcinogens (Dalbey *et al.*, 1980).

1.1.3.2 Radon gas

This indoor pollutant has recently attracted a lot of attention as a potential cause of lung cancer.

Radon is an inert gas formed from radium during the natural decay of uranium. The predominant source of radon in indoor air is the soil beneath structures. Radon diffuses through the soil and enters basements and from there spreads throughout the air in the home. Radon and its decay products are invariably present in indoor air, and a wide range of concentrations has been observed in homes. Some homes have levels comparable with those measured in uranium mines. But the majority are much lower (Harley, 1984).

Radon decomposes into short-lived particulate decay products which can attach themselves to environmental aerosols and be deposited in the bronchi. Two of these products emit alpha particles, which are highly effective in damaging cells because of their high energy and high mass. The effects of such radiation damage are cumulative and can eventually lead to lung cancer. Prolonged exposure to radon in Uranium miners has been shown to cause a characteristic codon 249 mutation in p53 gene which is quite distinct from the usual GC to TA transversions caused by tobacco smoke. In America extensive epidemiologic data from studies of miners has established a causal association between exposure to radon and lung cancer. There is synergism between the effects of cigarette smoke and radon particles implying that smokers are more susceptible to the cancer inducing effects of radon (Samet, 1993).

World wide radon is considered to be the second most important cause of lung cancer although its overall contribution is still negligible compared to cigarettes.

1.1.3.3 Atmospheric pollution

Many potential cancer-causing agents are released into the atmosphere from natural sources and from the industrial and non-industrial activities of humans. Polycyclic hydrocarbons are generated by fossil fuel combustion and industrial activities and can be identified in the air of urban locations. Other respiratory carcinogens include metals, radionuclides, diesel exhaust, and asbestos fibres. Unquestionably we all swim in a sea of carcinogens and it is conceivable that atmospheric pollutants may play some role in the increased incidence of bronchogenic carcinoma today. However the epidemiologic evidence for this is limited and the role of atmospheric pollutants, if any, is greatly subservient to that played by the personal pollution of tobacco smoke (Vena, 1982).

It may be that environmental agents act as co-carcinogens by augmenting the effects of other carcinogens. Thus animal experiments investigating the effects of two common gaseous pollutants (sulphur and nitrogen dioxides) and one common particulate pollutant (ferric oxide) have found that none of these agents is carcinogenic alone but they are capable of producing a carcinogenic effect from known carcinogens at doses that would otherwise be ineffective (Nettesheim *et al.*, 1975).

1.1.3.4 Occupational factors

Certain occupations are associated with a higher than expected incidence of lung cancer. Often the causative agent is known but the additive or multiplicative effect of cigarette smoking makes the apportionment of blame difficult in individual cases. Asbestos is particularly notable for its association with lung cancer in addition to causing diffuse pulmonary fibrosis. Non-smoking asbestos workers have a 5-fold increased risk of death from lung cancer as compared with other non-smoking workers. Asbestos workers who smoke one pack of cigarettes per day experience a 90-fold increased risk as compared to unexposed non-smokers. In this respect the effects of smoking and asbestos are multiplicative (Selikoff *et al.*, 1968; Berry *et al.*, 1972).

Increased risks of lung cancer have been described in workers extracting metal ores from deep mines. The causative agent in these cases is radioactivity from radon gas rather than injury by the metal being sought (Harley, 1984). Other occupational exposures associated with lung cancer include nickel refining, the extraction and production of chromium salts, the use of arsenicals in the metal refining and chemical industries, the use of chloro-ethers in various organic industrial processes and coke oven working in the steel industry. It is suspected that printing ink used in the newspaper industry may have been associated with lung cancer.

1.1.3.5 Other diseases of the lung

Localised scarring of the lung and diffuse pulmonary fibrosis tend to predispose to lung cancer. Scarring, the consequence of inflammation, may promote carcinogenesis by providing a local environment rich in factors that stimulate cell proliferation and

angiogenesis (see Section 1.1.4.4 a). Histologically such scar associated lung tumours are usually adenocarcinomas. Microscopically areas of epithelial hyperplasia are often seen in the near vicinity of lung scar tissue. These may in time undergo neoplastic change. However the mere presence of a scar is not enough to explain the development of these tumours and the patient's smoking history is also highly relevant in these cases. This is illustrated by the relationship between asbestos and smoking discussed above (Section 1.1.3.4). Fibrosing alveolitis whether cryptogenic or occurring with a connective tissue disorder such as systemic sclerosis or rheumatoid disease is also associated with an increased incidence of adenocarcinoma (Turner-Warwick *et al.*, 1980). The association of adenocarcinoma and old TB scars has already been mentioned in Section 1.1.2.2.

Chronic bronchitis and emphysema (both smoking related diseases) may represent independent risk factors for the development of lung cancer, even when age, gender and smoking history have been considered (Skillrud *et al.*, 1986).

1.1.3.6 Diet

Diet has recently been considered a potential influence on lung cancer risk in smokers. Nutrients of particular interest include Vitamin A, Vitamin C, Vitamin E and selenium. Data is sparse and inconclusive on all except Vitamin A (Colditz *et al.*, 1987).

Vitamin A is a fat soluble vitamin found mainly in animal derived foods e.g. eggs, milk, meat and fish. Vitamin A precursors called carotenoids (e.g. beta-carotene) are found abundantly in leafy green and yellow vegetables e.g. spinach and carrots. Vitamin A is thought to play a role in cell differentiation and proliferation by binding to nuclear receptors that regulate gene transcription. Deficiency of Vitamin A causes deregulated cell growth which leads to squamous metaplasia of specialised epithelium in the upper respiratory tract and the gastro-intestinal tract. It is speculated that advanced metaplasia can, in time, lead to cancer at these sites. In addition to this direct role in cell proliferation, Vitamin A (along with some other Vitamins) may be able to prevent cancers by acting as an anti-oxidant within the cell. Epidemiologic data show a protective effect of dietary Vitamin A intake from vegetable sources, but not of pre-formed Vitamin A which is derived from meat and dairy sources.

High dose Vitamin A has been used to successfully prevent the development of second primary tumours in patients with 'cured' head and neck cancers which is similar to lung cancer in many aspects. Similar trials are underway to prevent lung cancer in high risk groups with chronic administration of high dose Vitamin A (Hong *et al.*, 1990).

1.1.4 Lung Carcinogenesis

1.1.4.1 General Remarks

It is now widely accepted that carcinogenesis is a multistep process characterised by the stepwise acquisition of various heritable genetic defects. These genetic changes cause the cell to undergo a series of morphological, biochemical and physiological alterations until it finally becomes frankly malignant. This process can take years and during this intervening period the tissue becomes increasingly abnormal in appearance and behaviour. Epithelial tissues, for instance, go through the stages of hyperplasia, dysplasia, metaplasia, adenoma and carcinoma *in situ* before the appearance of invasive carcinoma.

The transition from pre-malignancy to malignancy has been most closely studied in the colon where a large percentage of patients with benign adenomas eventually go on to develop colorectal cancer. The existence of this clinically recognisable pre-malignant stage has enabled the molecular events of colorectal carcinogenesis to be delineated in a way that is not possible for most other malignancies. Mutations in at least five to six growth controlling genes is necessary for the development of colonic cancer. There seems to be a preferred temporal order for these mutations and the appearance of each new mutation coincides with progression to the next higher stage of pre-malignancy (Vogelstein *et al.*, 1988).

Although a similar series of events can be presumed to take place in lung cancer as well, it has been almost impossible to prove because of the practical difficulties implicit in subjecting an 'at risk' group to the hazards and discomfort of repeated bronchoscopic examinations. Also, most patients of lung cancer have invasive if not metastatic disease at presentation precluding any analysis of disease progression. Thus, most of our knowledge

in this field is obtained from circumstantial evidence (clinical studies correlating oncogene expression and disease outcome) and from extrapolation of molecular events seen in cell lines and in animal models of lung cancer.

Such drawbacks notwithstanding, it has been possible to piece together a composite picture of the events that most likely precede the onset of lung cancer.

1.1.4.2 Introduction

The process of conversion of normal cells to malignancy takes place in three stages. This was demonstrated by experiments on skin carcinogenesis in mice. Treatment of an area of the skin with low doses of methylcholanthrene-a genotoxic agent-“initiates” the disease but does not immediately result in the development of tumours. Subsequent chronic irritation with croton oil (itself not carcinogenic) leads to the formation of mostly benign papillomas. It seems that the initiated cells are “promoted” to a higher state of proliferation by this co-carcinogen. Further treatment with genotoxins results in malignant transformation of some papillomas-“tumour progression” (Berenblum, 1941).

A similar step-wise progression of events is seen in lung cancer. Individual cells within the tracheobronchial tree are “initiated” when they develop mutations after exposure to carcinogens in cigarette smoke. After a long latent period, when eventually the right set of conditions emerge, some of the initiated cells proliferate into small islands of pre-malignant clones-“tumour promotion”. Finally the cells become frankly malignant by acquiring new genetic defects. The stage of “tumour progression” is characterised by expansion of the tumour and acquisition of a more aggressive phenotype e.g. the ability to invade blood vessels and lymphatics and metastasise to distant organs.

1.1.4.3 Lung Tumour Initiation

Nine out of ten cases of lung cancer are caused by cigarette smoke which contains genotoxic carcinogens and is irritant (Harris, 1983). Other carcinogens like radon gas, atmospheric and industrial pollutants are rarely involved (see Section 1.1.3). The chief carcinogen in cigarette smoke is benzo(a)pyrene (BP) a polycyclic aromatic hydrocarbon. The mutations observed in several genes which are frequently altered in lung cancer are

those that would be expected from the chemistry of BP thus directly proving its pathogenicity. But before BP can induce mutations in genes it needs to be transformed to its active form within the cell. Individuals differ in their ability to metabolise and activate carcinogens which would explain the observed differences in susceptibility to cigarette smoke amongst individuals and families. Subjects who have a high level of aryl hydrocarbon hydroxylase (AHH) – one of the enzymes concerned with activating respirable hydrocarbon - show a greater propensity to lung cancer (Kellermann *et al.*, 1973). Similarly, the ability to metabolise Debrisoquin an anti-hypertensive agent may be an indirect indicator of the ability to activate carcinogens within cigarette smoke (Ayesh *et al.*, 1984).

The active form of the carcinogen forms adducts with DNA and induces mutations in particular genes. The cell has in-built defence mechanisms to detect and repair these genetic defects. If the defect is very severe and cannot be repaired the cell dies by apoptosis, a physiological process of cell death which is discussed in detail in the next section (Section 1.2). If, for any reason, these defences are breached, and cell division occurs before repair can take place, then any changes in one DNA strand will be replicated and no longer recognisable as an error. Thus the DNA mutation will be perpetuated and passed on to all future progeny.

It is not surprising therefore that one of the earliest genes to be inactivated in lung cancer is p53 a gene involved in modulating apoptotic cell death in response to DNA damage (see Section 1.2.3.5 b). Unlike colonic cancer where p53 mutations occur late in the process, in lung cancer such mutations are found even in pre-neoplastic lesions like dysplasia and metaplasia (Bennett *et al.*, 1993). In a retrospective analysis of sputum specimens stored over a 10-year period, evidence was found for mutations in p53 in eight of the ten patients who subsequently developed lung cancer. This has potential uses in the surveillance and screening of high risk patients e.g. heavy smokers (Sidransky, 1994).

Mutations of the p53 gene are a relatively common event in lung cancer being found in almost all SCLCs and in about 70% of NSCLCs. In the latter group, the highest incidence is amongst squamous cell carcinomas whereas only 30% of adenocarcinomas show p53 mutations (Takahashi *et al.*, 1989; Greenblatt *et al.*, 1994). Although it is an early event

the loss of p53 seems to have a bearing on the biological behaviour of the cancer in its later stages as well. Tumours with inactivated p53 show a higher rate of metastases to hilar nodes and distant sites (Fontanini *et al.*, 1994).

Another early event in 100% of SCLCs and 75% of NSCLCs is deletion in the 3p chromosome. Such a deletion, coupled with a mutation within a putative tumour suppressor gene located on chromosome 3p, could be another important step in the development of lung cancer. It is almost certain that the short arm of chromosome 3 contains a tumour suppressor gene crucial for the development of lung cancer. Multiple candidate tumour suppressor genes that map to the consistently deleted segment of chromosome 3p include retinoic acid receptor, protein tyrosine phosphatase gamma and the von Hippel-Lindau disease tumour suppressor gene. None of these have yet been proven to be the culprit (Brauch *et al.*, 1990).

Initiating mutations take place in large number of cells but only a small fraction survive and the final tumour probably results from the clonal expansion of only one of the initiated cells. The other surviving initiated cells remain dormant for years or undergo transformation at a much slower pace and may emerge as a second tumour years later. The high incidence of “recurrent” lung tumours includes a fair proportion of what should rightly be called second primary tumours which display genetic defects quite different from the initial tumour. This has given rise to the **Field Cancerization** theory which describes the diffuse fieldwise mucosal carcinogenic effects of tobacco. In fact this field is now thought to extend beyond the tracheobronchial tree and include the upper digestive tract and the head and neck as well. There is a high incidence of second primary tumours in the lung following successful treatment of cancers in these areas and it is likely that both tumours were initiated by the same inciting agent (Foulds, 1969; Chung *et al.*, 1993).

1.1.4.4 Lung Tumour Promotion

Once a cell has been initiated the next step is to expand the clone by accumulating faster than its neighbouring normal cells. This stage of tumour promotion can last for years and is clinically silent. During this phase the epithelium passes through a continuum of

histological changes that include hyperplasia, dysplasia and carcinoma *in situ*. It is unlikely that every initiated cell is promoted into a pre-neoplastic clone. To be successfully promoted a clone of cells must display a combination of the following properties:- (a) rapid proliferation rate (b) slow apoptotic rate (c) biochemical changes that favour proliferation.

(a) Rapid proliferation

_Initiated cells can multiply rapidly in response to **growth factors** Chronic inflammation is known to promote tumours at several sites in the body by maintaining a steady supply of growth factors and cytokines (Grasso, 1987). There is a high incidence of cancer in association with ulcerative colitis, chronic pancreatitis, chronic viral hepatitis and oesophagitis. In the lungs the chronic bronchial inflammation engendered by the constant inhalation of cigarette smoke is likely to create the right milieu for tumour promotion. Healing wounds, as in scars from old tuberculosis, have been shown to predispose to lung adenocarcinomas (1.1.2.2).

Although an excess of prevailing growth factors would help, it would still not place the initiated cells at any position of special advantage as the neighbouring normal cells too would be exposed to the same factors. There are two ways in which the initiated cells can get round this problem. They can either increase their sensitivity to these factors by expressing more growth factor receptors on their cell surface or they can manufacture their own growth stimulants. There is evidence to show that both these events happen during the stage of lung tumour promotion.

Non small cell lung cancers in general and squamous cell lung cancers in particular have been shown to over-express a spectrum **growth factor receptors** (Berger *et al.*, 1987). These include the epidermal growth factor receptor (EGFR)-encoded by the gene *c-erbB1*, transforming growth factor α (TGF α) receptor and a EGFR-related receptor for an unknown ligand coded by the *Her-2/neu* gene. It is interesting that none of these receptors are found on SCLC cells which are more adept at synthesising their own growth factors.

SCLC cells are known to produce **peptide hormones** with growth stimulating properties. They also express the corresponding receptors to complete the 'autocrine loop'. Examples include gastrin releasing peptide (GRP), bombesin, neuromedin B and insulin like growth factor I (IGF-I). Of these GRP is thought to be an important tumour promoter in early lung cancer (Johnson, 1995). During this stage of promotion, cells may be susceptible to negative influences as well –for example to retinoic acid – a fact which is utilised in the design of chemoprevention programmes (see Section 1.1.3.6).

It is likely, that as the cells become increasingly dysplastic they outgrow their dependence on these extrinsic growth stimulants.

(b) Slow apoptosis

Reduced loss of cells from preneoplastic populations might also be expected to influence their growth. There is evidence from animal models of possible interplay between proliferation and apoptotic cell death in early carcinogenesis (Section 1.2.5).

Two of the main genes involved in the control of apoptotic cell death i.e. p53 and bcl2 have been found to be deranged in lung cancer from a very early stage. Loss of p53 protein would make a cell less prone to apoptosis . As mentioned before this is seen to happen in a large fraction of both SCLCs and NSCLCs.

The product of the bcl2 gene inhibits apoptosis (see Section 1.3). Several studies have shown that about 30% of NSCLCs overexpress Bcl2 and the observations of Pezzella *et al.* indicate that such overexpression may start from a very early stage (Pezzella *et al.*, 1993). The role of bcl2 in lung cancer is quite complex and extends beyond its role as an anti-apoptotic agent. This is discussed more fully elsewhere (see Section 6.8).

During tumour promotion cells which are less prone to apoptosis because of derangement of either or both of these genes are theoretically more likely to survive and acquire more harmful genetic mutations.

(c) Biochemical changes

A whole host of subtle biochemical changes have been reported in pre-malignant cells in the bronchus and elsewhere. These include shifts in the pentose phosphate and glycolytic

pathways. Such changes might be expected to endow pre-neoplastic cells with the ability to channel more of their energy production into processes like cell proliferation (Ito *et al.*, 1995).

1.1.4.5 Lung Tumour Progression

The stage of tumour progression is characterised by accumulation of yet more mutations in oncogenes and anti-oncogenes. There is controversy about the minimum number of such mutations that is necessary with estimates ranging from six to twenty. There is controversy also about the need for these mutations to occur in any particular order. While the Vogelstein model of colorectal cancer seems to imply that an orderly progression is necessary most other commentators agree that the 'bulk' of the mutations is more important than the order in which they are acquired. Yet certain genes tend to be mutated more often at certain stages of the disease. There is controversy about whether these later genetic events occur 'endogenously' i.e. as a result of the inherent genetic instability within the cell or whether further carcinogen exposure is needed. Most lung cancer patients continue to be exposed to cigarette smoke carcinogens till a late stage of their disease and even the later genetic mutations in such patients show characteristic GC to TA transversions.

As a result of the genetic alterations during the stage of progression, the tumour becomes more aggressive and acquires the potential to invade local tissues and spread to distant organs. Metastasis is the result of a multistep process including basement membrane disruption, stromal invasion, neoangiogenesis, intravasation and extravasation of tumour cells and invasion of a secondary site. Thus several aspects of normal cell behaviour must be disrupted before a cancer cell is able to metastasise successfully.

Resistance to apoptosis should be an advantage to a metastasising cancer cell. A lung cancer cell in the alien environment of a lymph node or liver parenchyma is deprived of many survival signals and the normal reaction is to undergo apoptosis. Cells which are inherently resistant to apoptosis (because of the appropriate genetic mutations) should find it easier to survive and colonise a distant organ (see Section 1.2.5.3).

An example is the resistance to apoptosis which is conferred by the loss of expression of the DAP (death associated protein) kinase gene. DAP kinase is a calcium/calmodulin dependent enzyme that was first identified as a positive modulator of apoptosis induced by IFN- γ in HeLa cells (Deiss *et al.*, 1995). Inactivation of the DAP kinase gene may be an important event in the later stages of lung cancer progression. Highly aggressive and metastatic murine lung cancer clones were found not to express DAP kinase in contrast to their low metastatic counterparts. Restoration of DAP kinase to physiological levels in high metastatic Lewis carcinoma cells suppressed their ability to form lung metastases after intravenous injection into syngeneic mice and delayed local tumour growth in a foreign micro-environment (Inbal *et al.*, 1997).

Alteration in the matrix metalloproteinases (MMPs) is observed late in the life history of many tumours and seems to correlate with enhanced metastatic potential. The MMPs are a family of proteases capable of degrading the components of basement membrane and extracellular matrix. It is obvious that such enzymes will have enormous practical value to metastasising cells. Normal tissues also produce inhibitors of MMPs called TIMPs (tissue inhibitors of metalloproteinases). In a study involving 88 resected lung tumours where cancerous areas as well as adjacent normal lungs were examined for expression of MMPs and TIMPs, it was observed that progression of bronchopulmonary cancers involves a progressive disruption of the MMP/TIMP balance in favour of MMPs. High MMP expression correlated with poorer histological differentiation and higher clinical stage. These enzymes may represent an attractive target for anti-invasive and anti-metastatic therapy for lung tumours (Nawrocki *et al.*, 1997).

Acquisition of metastatic capacity has also been associated with reduced expression of the nm23 gene. This gene codes for a nucleoside diphosphate kinase which is active in microtubule assembly. Mitotic spindle formation and cell locomotion both require microtubule function and are both deranged in malignant cells. In NSCLC the immunohistochemical expression of nm23 has been found to correlate inversely with the frequency of distant metastases. This antibody may be useful in selecting patients for post-operative adjuvant chemotherapy (Lai *et al.*, 1996).

CD44 is an integral membrane glycoprotein that functions as a receptor for the extracellular matrix glycan, hyaluronan. Expression of CD44 isoforms by tumour cells is thought to play a role in tumour growth and metastasis by altering the anchorage properties of malignant cells to other cells and to basement membrane (Horn *et al.*, 1996).

A number of other genes also participate in lung tumour progression (reviewed by Gazdar, 1994). Ras is involved primarily in pulmonary adenocarcinomas. Activation of myc is associated with a greater incidence of lymph node and distant metastases in squamous cell carcinoma. The retinoblastoma gene is absent or abnormal in 20% of NSCLCs and influences disease progression.

In the late stages a range of **karyotypic abnormalities** can be observed in lung cancer including loss of 9p and 11p (Testa and Siegfried, 1992). Some of these regions might host genes of importance (such as the interferon gene on 9p) but it is still not clear whether these deletions have any pathological significance or whether they are merely a reflection of the state of genetic anarchy that prevails in cells at this late stage.

From the foregoing discussion it can be seen that the failure of apoptosis is an important event in the early stages of the natural history of lung cancer and it is likely to play a crucial role in the late stages as well for instance during metastasis. The role of apoptosis in cancer (and in other pathological processes) is being investigated enthusiastically in laboratories around the world. In the next section we shall describe this newly discovered biological phenomenon and explain why its discovery has engendered so much enthusiasm and optimism amongst biologists and physicians alike.

1.2 Apoptosis

1.2.1 Introduction

Evidence for the existence of two morphologically distinct types of cell death was first obtained by Kerr in 1965 from histochemical studies of ischaemic injury to rat liver (Kerr, 1965). Some cell death occurred with the typical changes seen in tissue necrosis: clumping of chromatin into ill defined masses, swelling of organelles, membrane disintegration and infiltration of inflammatory cells. Cells in other areas of the damaged liver, however, died a different death. They contained chromatin compacted into sharply delineated masses, the cytoplasm was condensed, and there were outcropping of cytoplasmic “blebs” or protuberances that became pinched off and released to be devoured by tissue phagocytic cells. No inflammatory reaction was noted around cells dying by this second mechanism. Kerr’s further studies with Currie and Wyllie showed that this second mechanism of cell death occurred as tissues underwent remodelling during development and in that sense was a “physiologic cell death” (Kerr *et al.*, 1972). The original term for this phenomenon, “shrinkage necrosis”, did not seem an appropriate one for this process, so they searched for another. A colleague of theirs at the University of Aberdeen, professor James Carmack of the Department of Greek, suggested the term *apoptosis* meaning “falling off” of petals from a flower or leaves from a tree. The term has stuck ever since.

The concept of apoptosis was largely ignored till the mid to late 1980s when the discovery of the *ced* genes in the roundworm *Caenorhabditis elegans* and of the *bcl2* gene in B lymphocytes put the field on a solid genetic basis. In spite of extensive research the molecular processes controlling and executing cell death through apoptosis are still poorly understood. Apoptosis has been shown to be involved in diverse biological processes both physiological and pathological. In particular the role of apoptosis in cancer has been investigated with great zeal and fervour.

Recognition of the widespread importance of apoptosis has been one of the most significant changes in the biomedical sciences in the past decade.

1.2.2 Morphological features

The morphological changes of apoptosis are quite distinctive and can be said to occur in three phases (Wyllie *et al.*, 1980).

Phase 1.

The earliest ultrastructural changes of apoptosis include the loss of cell junctions and other specialised plasma membrane structures such as microvilli. This causes the cells to detach themselves from their neighbours and adopt a smooth contour.

The most characteristic change occurs next and consists of condensation of chromatin into crescentic caps at the nuclear periphery. This change is associated with cleavage of nuclear DNA.

Along with these nuclear changes the cytoplasm gradually condenses, the organelles become compacted and the cell generally shrinks in size (Fig. 1.2). The contraction of cytoplasmic volume is associated with loss of intracellular fluid and ions. This is the result of the endoplasmic reticulum dilating and forming vesicles that fuse with the plasma membrane and void their contents extracellularly. Cytoskeletal elements aggregate in side-to-side arrays often parallel to the cell surface and ribosomal particles clump in semi-crystalline formations but otherwise the organelles remain intact. In contrast to necrosis mitochondria do not show high amplitude swelling, the cell membrane does not become permeable to vital dyes at this stage and apoptotic cells within tissues do not elicit an acute inflammatory reaction. These alterations in cell volume and shape have been ascribed in part to the induction in apoptotic cells of the enzyme transglutaminase (Section 1.2.3.4 c).

In phase 2 (which may overlap with the first) there is blebbing at the cell surface and crenation of the nuclear outline. Both nucleus and cytoplasm may split into fragments of various sizes. Typically the cell becomes a cluster of round smooth membrane bound apoptotic bodies some containing nuclear fragments and others without. Apoptotic bodies provide a potent stimulus for phagocytosis and are engulfed by neighbouring cells. These engulfing cells are often members of the mononuclear-phagocyte system but also can be normal epithelial cells, vascular endothelium and tumour cells.

In phase 3, there is progressive degeneration of residual nuclear and cytoplasmic structures. In tissues these changes (sometimes called secondary necrosis) usually occurs in the phagosomes of the ingesting cell. Eventually membranes disappear, organelles become unrecognizable and the appearance is that of a lysosomal residual body.

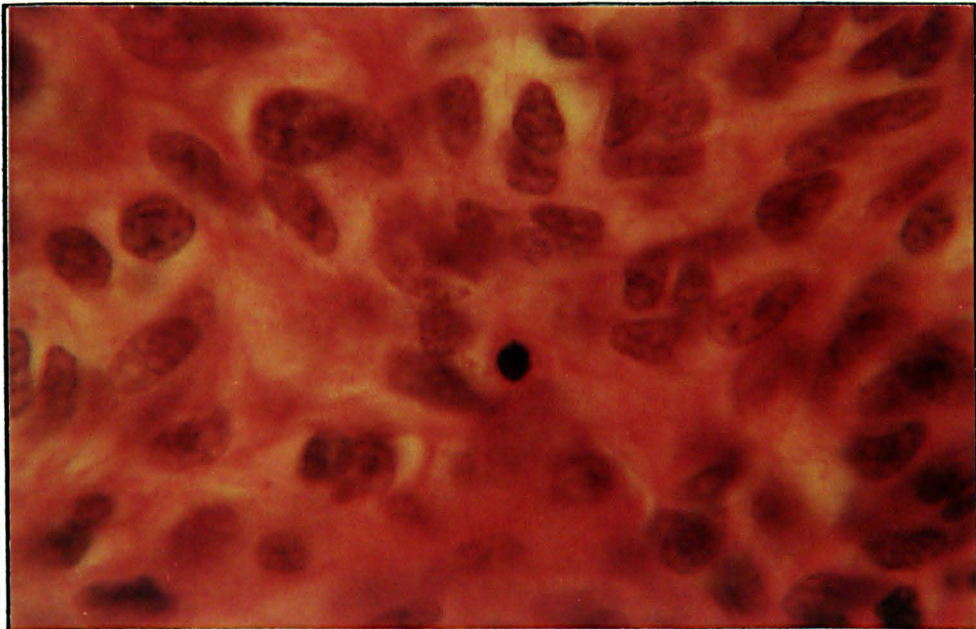


Fig. 1.2 Section of squamous cell lung cancer ($\times 630$) stained with H&E showing an apoptotic cell characterised by dark pyknotic nucleus and condensed cytoplasm.

A brief word about the **speed of apoptosis**. Time lapse cinematographic studies have shown (Evan *et al.*, 1992) that the initial changes of apoptosis (Phases 1 & 2) are achieved in the course of a few minutes and results in the formation of small dense apoptotic cells. If not phagocytosed immediately, these cellular particles undergo a gradual loss of cell density but remain recognisable within tissues for 3-6 hours before they are completely lysed by secondary necrosis. This rapidity of clearance of apoptotic bodies *in vivo* means that the identification of only a few apoptotic bodies in a tissue section can represent a considerable degree of cumulative cell loss. Numerically small differences in 'apoptotic indices' (i.e. the percentage of cells that are apoptotic) can therefore be of great

biological import. For example, an intravenous bolus of anti-CD4 antibody increases the apoptotic index in murine lymph nodes from 0.06% to 1.33%, and this is sufficient to halve the total cell count of the lymph nodes within 48 hours (Howie *et al.*, 1994).

1.2.3 Biochemical mechanisms and genetic control of apoptosis

1.2.3.1 Introduction

A close inspection of the phenomenon of apoptotic cell death reveals that there are four components to it. First of all there must be a **signal**, arising either from within the cell or outside it, which asks the cell to commit suicide. This message is conveyed via **second messengers** or signal transducers to the **death effectors**—a group of chemicals which when activated kills the cell producing the typical morphological features of apoptosis. Finally there are **modulators** which can (positively or negatively) influence the activity of the effectors and transducers.

1.2.3.2 Signals

Apoptosis can occur in response to an external or internal trigger (Bellamy *et al.*, 1995). Apoptosis is commonly seen during an organism's development and is best exemplified by the co-ordinated death of web space cells that sculpts digits from the coarsely shaped limb bud and by the chronologically and spatially invariant death of 131 cells that occurs during development of the nematode *Caenorhabditis elegans* (Ellis *et al.*, 1991). These examples of developmental apoptosis were previously thought to be 'autonomous' in that no trigger was apparent and cell death was presumed to be initiated by some internal clock or by an in-built 'programme' within the genome which caused specific cells to die at specific times. However, it has now been found that signals like retinoic acid trigger apoptosis in many of these situations for instance in the limb bud (Jiang and Kochhar, 1992) and in the developing brain (Maden *et al.*, 1997).

In other situations there are clearly identifiable triggers arising from within the cell. Examples include DNA damaged by radiation or chemotherapy or foreign (viral) nucleic acids within the cytoplasm.

External stimuli can be (a) appearance or withdrawal of soluble signalling molecules like growth factors, cytokines or hormones (b) cell bound ligands e.g. Fas ligand or (c) matrix associated molecules that act on surface integrin receptors. The latter is now recognised as an important survival signal for differentiated endothelial and epithelial cells. Without integrin-matrix binding these cells undergo apoptosis, a phenomenon that has been termed anoikis ('homelessness'). Such cells therefore cannot survive out of position if the appropriate matrix requirements are not fulfilled. Thus matrix composition localises seed to soil in a strict manner, a concept of critical importance to understanding mechanisms of neoplastic progression (see Sections 1.1.4.5 and 1.2.5.3c). In contrast fibroblasts do not show integrin dependence, in keeping with the need to rove across tissue boundaries during repair of injury (Meredith *et al.*, 1993; Frisch and Francis, 1994).

A cell is constantly being showered by such external chemical information which it must interpret in the context of its own lineage, developmental stage, cell cycle stage and metabolic state. After integrating all this information there are basically four ways in which a cell can respond. It can maintain the *status quo* of quiescence, it can divide, it can differentiate (if it is an immature cell) or, if it senses that its presence is redundant or harmful to the organism, it can decide to die by apoptosis (Williams and Smith, 1993). The same stimulus can evoke different responses in different cells at different times. For example, all the cells in the body of a metamorphosing tadpole are exposed to increased levels of thyroid hormone. In response to this the developing limb differentiates to produce legs, while muscle cells in the tail die by apoptosis.

Thus there probably are no specific "apoptotic signals" as such. The context in which a signal arrives (or is withdrawn) largely determines its effect on the cell.

1.2.3.3 Signal Transduction

Signals generated within or outside the cell must be transmitted to downstream effector molecules that often lie in different subcellular compartments. Of the various signal transduction systems two are known to be heavily involved in apoptosis.

(a) Calcium-phosphoinositol-PKC

Following the binding of a wide range of hormones and growth factors to their receptors there is activation of phospholipase-C γ (PLC γ) which enzymatically cleaves membrane phosphatidylinositol biphosphate (PIP₂) to produce diacyl glycerol (DAG) and inositol trisphosphate (IP₃). DAG operates within the cell membrane to activate protein kinase C (PKC) and IP₃ mobilises calcium from cellular stores like the endoplasmic reticulum (ER) to raise the intra-cellular free calcium level. Some of this free calcium after complexing with a protein called calmodulin activates several enzymes of the PKC family. The substrates of PKC include cytoskeletal proteins, cell surface receptors and other proteins and enzymes which may form part of the apoptotic machinery. In addition calcium may directly activate enzymes such as endonuclease and transglutaminase which have a more obvious role in apoptosis (Hale *et al.*, 1996) .

Sustained increases in intracellular free calcium precede apoptosis induced by a number of agents or conditions, and apoptosis is delayed or inhibited when calcium is depleted from the cellular growth medium. Calcium ionophores such as A23187, which allows calcium to enter the cell, induces apoptosis in some cell types. However, calcium influx does not accompany apoptosis in all cell types and thus may not be an essential requirement but rather an after-effect of cell membrane perturbation.

Increased levels of the mRNA encoding the calcium-binding protein calmodulin have been observed in apoptotic cells, and calmodulin antagonists inhibit apoptosis in some cell types (Zheng *et al.*, 1991). Evidence for the effects of protein kinase C activators and inhibitors is equivocal. Phorbol esters, which stimulates PKC, inhibit apoptosis in some cell types, but inhibition of PKC inhibits apoptosis in others (Schwartzman and Cidlowski, 1993).

Thus it is likely that this pathway of apoptotic signal transduction is used only in some cells in response to specific signals.

(b) Ceramide

Ceramide is used as an apoptotic signal transducer by Fas (also known as Apo1 or CD95) a member of the tumour necrosis factor receptor (TNFR) superfamily (see Section 1.4.1). When Fas is bound by its ligand, Fas-L, the cell dies by apoptosis. It has been shown that Fas binding leads to the enzymatic cleavage of membrane sphingomyelin to form ceramide. Ceramide triggers the subsequent stages of the apoptotic pathway perhaps by activating the ICE/ced-3 cysteine proteases discussed in Section 1.2.3.4 a. Inhibition of ICE/ced-3 by the cowpox virus protein CrmA can prevent Fas and ceramide mediated apoptosis.

Ceramide has also been found to be involved in several other apoptotic pathways e.g. that following dexamethasone, cross-linking of surface receptors by antibodies and serum withdrawal (Pushkareva *et al.*, 1995).

The internal signal of DNA damage (induced by radiation or chemicals) is transmitted to the death effector machinery by the p53 gene product. In that sense it too is a signal transducer (see Section 1.2.3.5 b). Several other such signal transduction pathways exist e.g. cAMP and its analogues or agents that increase intracellular cAMP level, and the cytoplasmic glucocorticoid receptor which transduces dexamethasone induced apoptosis. Often these paths cross each other so that one signal can modulate the effects of another.

1.2.3.4 Cell Death effectors

There are two ways in which signal transducers may set into motion the death effector machinery within a cell. They may activate the transcription of genes which code for these effectors. Alternatively, they may activate pre-formed death effectors within the cytoplasm. It seems that the second alternative is more commonly practised. While in some instances inhibitors of RNA and protein synthesis have been found to inhibit apoptosis in most other cases their presence has no effect on the process and may even

enhance apoptosis (Raff *et al.*, 1993). Also cells from which nuclei have been removed are capable of dying apoptotically indicating that new protein synthesis is not essential for this process (Jacobson *et al.*, 1994).

Although the full range of such molecules is unknown it seems that the same final set of molecules is universally used by all cells because the morphological changes of apoptosis are the same irrespective of species, cell type or trigger. Because these morphological changes can be prevented almost universally by a single protein - Bcl2 - it is probable that only a few effectors are involved in the final common pathway of apoptosis. Several candidates have been proposed.

(a) The ICE/ Ced-3 proteases or Caspases

These proteolytic enzymes are a core component of the cell death effector machinery.

Evidence for their involvement has arisen from studies on the nematode *Caenorhabditis elegans*. During the maturation of this worm apoptosis takes place in some cells in an orderly and predictable fashion making it an ideal candidate to study this phenomenon in great depth. So far 14 genes controlling apoptosis in *C. elegans* have been identified. Two of these Ced-3 and Ced-4 are involved in the actual implementation of cell death as mutations in these genes prevents apoptosis altogether. A third gene Ced-9 (to which bcl2 shows close structural and functional similarity) is a negative regulator of the activity of Ced-3 and Ced-4 (Hengartner *et al.*, 1992).

Cloning of the *C. elegans* Ced-3 gene showed that the protein is homologous to the mammalian cysteine protease ICE (Interleukin 1-B Converting Enzyme). The only known function of ICE is the proteolytic maturation of pro-interleukin 1-B to the biologically active inflammatory cytokine. Although this initially implicated ICE itself in mammalian apoptosis it now seems clear that other ICE homologues are more likely candidates as functional counterparts of Ced-3 in higher organisms. This has been supported, for example, by ICE deficient knockout mice where no defects in apoptosis appear.

Molecular cloning has identified several human homologues of ICE and Ced-3 including ICE_{rel}-II (TX, ICH-2), ICE_{rel}-III, ICH-1 (equivalent to murine Nedd2), CPP32 (apopain, Yama), Mch2 and Mch3 (ICE-LAP3). These enzymes are now collectively known as

caspases. Unlike other mammalian cysteine proteases, caspases cleave their substrates following aspartate residues. Furthermore proteases belonging to this family exist as zymogens that in turn require cleavage at internal aspartate residues to generate the two subunit active enzyme. As such, family members are capable of activating each other.

It has been shown for several of the caspases that over-expression can cause apoptosis and that death can be inhibited by interfering with protease function. The cowpox virus protein CrmA which is a specific inhibitor of ICE like cysteine proteases, can inhibit apoptosis in a wide range of situations indicating that caspases are recruited as effectors by a wide range of apoptotic stimuli. This recruitment does not require gene transcription or new protein synthesis as it has been shown to occur even in cells from which nuclei have been removed. This would imply that the caspases are always present in the cytoplasm awaiting activation.

Once activated these proteases act on a variety of substrates both in the nucleus and in the cytoplasm not all of which are needed for apoptosis. For instance, CPP32/apopain, which appears to play a key role and may be a human counterpart of nematode Ced3, cleaves the protein poly (ADP-ribose) polymerase (PARP). Although this is a convenient marker for apoptosis, cleavage of PARP is not actually related to cell death. Of more relevance is cleavage of nuclear lamins which is an early event in apoptosis. Lamins are intermediate filament proteins residing at the nuclear envelope that serve to organise chromatin within the nucleus. Inhibition of lamin cleavage can prevent many of the nuclear events of apoptotic cell death (Vaux and Strasser, 1996; Nicholson, 1996).

It is likely that apoptosis results from the downstream effects of several such cysteine protease cleaved substrates. Bcl2 is able to prevent apoptosis by blocking, in some unknown way, the various actions of the caspases (see Section 1.3.5).

(b) Endonuclease

The formation of DNA fragments of oligonucleosomal size (180-200 base pairs) is a biochemical hallmark of apoptosis in many cells and leads to the characteristic ladder pattern on gel electrophoresis (see Section 3.1.3). DNA cleavage is the result of an endogenous neutral Ca^{2+} and Mg^{2+} dependent endonuclease capable of inducing double

strand breaks at internucleosomal sites. Recent observations also suggest that large DNA fragments (30-50 and 200-300 kb) occur during cell death. Such large fragments may not be precursors of the oligonucleosomal fragments and both types of fragments can be produced, apparently independently, under some circumstances (Hale *et al.*, 1996).

Several candidate endonucleases of different cellular origins have been reported e.g. DNase I, DNase II and Nuc 18. These endonucleases are present constitutively in some cells (e. g. thymocytes) where they are activated by a rise of free cytosolic calcium, whereas in others the enzyme is induced transcriptionally before the onset of apoptosis.

(c) Transglutaminase

The characteristic alterations in cell volume and shape seen in apoptosis have been ascribed in part to the induction of transglutaminase, a calcium dependent enzyme which causes extensive cross-linking of cytoplasmic proteins forming a shell under the plasma membrane. This stabilizes the cytoplasm of dying cells, preventing the leakage of harmful intracellular elements into the extracellular environment which could lead to an inflammatory reaction. Five transglutaminases have been identified in humans.

Neither endonuclease or transglutaminase is absolutely indispensable and apoptosis has been shown to proceed even in their absence. These enzymes are activated after the cysteine proteases and it seems that they are not the actual killers but are merely involved in the post-mortem packaging of dead cells.

1.2.3.5 Modulators

Whilst the triggers and the effectors of apoptosis are being defined, it is clear that there are other factors which may modulate the process.

The modulation of apoptosis is complex and the details are still being unravelled with new bit players coming to light every day. The main genes controlling apoptosis are bcl2, p53 and c-myc. While the latter two are called into play only in certain cell types and in response to certain special situations, bcl2 or one of its homologues is part of the hard wired pathway of cellular apoptosis in most cells in all species from worms to humans.

(a) Bcl2

Bcl2 is the prime controller of apoptosis (see Section 1.3). Most other modulators (including p53 and c-myc) are able to control apoptosis mainly by regulating the bcl2 homologues within a cell.

Over-expression of bcl2 inhibits apoptosis in most cells induced by most stimuli. How bcl2 achieves this is still not absolutely certain but all indications are that its ability to oppose the effects of the cysteine proteases is crucial. The mechanism by which Bcl2 opposes the caspases is far from clear. No direct chemical interaction between these two groups of molecules has been demonstrated or even proposed. But Bcl2 over-expression in a cell can prevent ICE induced apoptosis. That it can do so even in enucleated cells is proof that it does not act by down-regulating the genetic expression of ICE/ced-3 proteases but rather by opposing their effects in the cytoplasm. Thus two possibilities remain: bcl2 could either prevent the activation of the cysteine proteases from their precursors or block their actions after activation (White, 1996).

It has been known for some time that Bcl2 is but one member of a family of proteins which have different effects on apoptosis. Thus while Bcl2 and Bcl-x_L inhibit apoptosis Bax, Bcl-x_S and Bad promote it. These family members try to neutralise each others effects by forming heterodimers. Thus, Bax combines with Bcl2 to negate its anti-apoptotic effect. If Bax is in excess the cell undergoes apoptosis. If Bcl2 is in excess it lives. Similar interactions occur between other family members. Therefore it is the ratio of these various Bcl2 proteins within a cell that largely determines its response to an apoptotic stimulus. Several modulators like p53 and c-myc are capable of altering this ratio by altering the rate of synthesis of individual family members.

Yet another way of controlling Bcl2's action is by phosphorylating the proteins after they have been synthesised. This can inactivate them or alter their binding capacity to other Bcl2 homologues. Ras, which has been found to co-precipitate with Bcl2 within cells probably inactivates it by phosphorylation (Haldar *et al.*, 1995).

(b) p53

The pro-apoptotic p53 gene product prevents the proliferation of cells with damaged DNA. When U.V. rays or X-rays cause strand breaks in DNA the cellular concentration of P53 protein increases and this results in the cell cycle being blocked at G1. The cell is thus given time to repair the damage before the cell cycle is initiated again. If the damage is irreparable the cell is eliminated by apoptosis.

All these functions of p53 are subserved indirectly through other proteins. P53 is a transcriptional regulator of genes. It can increase or decrease the synthesis of several proteins by binding to the regulatory regions of their genes. For instance, p53 brings about cell cycle arrest by increasing the synthesis of the protein p21/WAF-1/CIP-1 and it aids DNA repair by increasing the synthesis of GADD 45 (Velculescu and El-Deiry, 1996).

Apoptosis is promoted by p53 mainly by altering the Bax:Bcl2 ratio within a cell. When activated p53 increases the synthesis of the pro-apoptotic Bax and decreases the synthesis of Bcl2. This explains why over-expression of Bcl2 can prevent p53 mediated apoptosis (Hoffman and Liebermann, 1994). p53 also upregulates the expression of the surface receptor Fas. Of course this will lead to apoptosis only if there is enough Fas-L around to bind to it.

The role of p53 in apoptosis is confined to special situations. It is needed for radiation induced but not dexamethasone induced apoptosis of lymphocytes. It is not needed for the programmed cell death that takes place during embryogenesis and therefore p53^{-/-} mice develop normally and are indistinguishable from other mice, except that from a young age these mice are prone to developing tumours at various locations. Thus the only physiological role of p53 seems to be tumour prevention.

(c) C-myc

C-myc too is a nuclear transcription factor that controls cellular functions by regulating target genes.

C-myc promotes cell proliferation. Following mitogenic stimulation there is increased synthesis of c-myc which leads to the transactivation of a set of genes thought to be essential for progression through G1. C-myc concentration within cells decline rapidly

following withdrawal of serum or specific growth factors and the cells accumulate in the G0/G1 phase.

Paradoxically c-myc can also lead to cell death by apoptosis. Enforced over-expression of c-myc in the absence of serum or growth factors has been shown to cause apoptosis in myeloid progenitor cells and fibroblasts among other cell types. How does c-myc cause these diametrically opposite effects? There are two possible answers to this riddle (Ryan and Birnie, 1996).

The so called “conflict model” proposes that apoptosis results from conflicting proliferative messages. While the lack of growth factors sends a negative growth signal, simultaneous over-expression of c-myc sends a positive growth signal. In the ensuing confusion the cell dies.

By contrast, in the “dual signal” model apoptosis is considered a normal outcome of c-myc expression which is evident only when there is a deficiency of growth factors. This system might have evolved as a fail-safe measure to eliminate cells with inappropriately activated c-myc.

How c-myc promotes apoptosis is not known. One proposal is that it upregulates p53 which in turn alters the Bax:Bcl2 ratio to cause apoptosis. This would explain why the pro-apoptotic effects of c-myc can be blocked by over-expression of Bcl2. However, c-myc can accelerate apoptosis in M1 leukaemic cells which lack p53 indicating that this might not be the only mechanism. Other mechanisms which have been proposed include over-expression of ornithine decarboxylase (ODC) which can kill cells by generating excess reactive oxygen species (ROS) (Peckham and Cleveland, 1995).

C-myc probably plays a part in the apoptosis which is seen during embryogenesis. Deletion of c-myc in mice is lethal in utero probably due to a physiologic deficit in the myc dependent deletion of cells. Many forms of apoptosis are independent of c-myc function.

(d) Other genes

A number of other gene products are implicated in the control of apoptosis e.g. ras, raf, fos, jun and rel. In most instances it remains to sort out primary regulators from secondary perturbations.

1.2.4 Apoptosis in health and disease

It is now widely accepted that apoptosis is of central importance for the development and homeostasis of metazoan animals. Aberrant apoptosis has been implicated in various pathological conditions.

1.2.4.1 Physiological role of apoptosis

Apoptosis is important in several physiological processes (Ueda and Shah, 1994).

Apoptosis serves as a prominent force in sculpting the **developing organism**. It is responsible for the regression of the tadpole tail that takes place during its metamorphosis into a frog and for removal of interdigital webs during limb development in mammalian embryos.

Apoptosis is a major mechanism for the **precise regulation of cell numbers**. In adult mammals, apoptosis occurs continually in slowly proliferating cell populations, such as epithelium of liver, prostate and adrenal cortex, and in rapidly proliferating populations, such as the epithelium lining intestinal crypts and differentiating spermatogonia. Although much of the cell loss in populations of the later type clearly is the result of shedding of cells from the tissue, in the former, mitosis and apoptosis balance each other under steady-state conditions. There is growing evidence that apoptosis is regulated in a reciprocal fashion to mitosis by growth factors and trophic hormones, and Raff *et al.* have suggested that most cells in higher animals may require continuous trophic stimulation to survive. They postulate that an increase in cell numbers in a particular location might lead to greater cellular competition for the trophic factors that stimulate mitosis and inhibit apoptosis and that this, in turn, might temporarily tip the balance between the two processes, leading to restoration of the cell population to its former level (Raff *et al.*, 1993).

A number of **involutional processes** occurring in normal adult mammals have been shown to be associated with marked enhancement of apoptosis. Well documented examples include reversion of the lactating breast to its resting state after weaning, ovarian

follicular atresia, and catagen involution of hair follicles. The trigger for breast involution is likely to be hormonal, but in the other instances, the nature of the initiating stimulus is uncertain.

In the **immune system**, apoptosis subserves special physiologic roles. It is responsible for the deletion of autoreactive T-cells in thymus that is responsible for self-tolerance and for selection of B-cells in lymphoid germinal centres during humoral immune responses. Another specialised function is the deletion of effete cells such as ageing neutrophil leukocytes.

Apoptosis is involved in **antiviral defence** mechanisms. There is good evidence that cells infected by viruses die through apoptosis before the virus can replicate. Interestingly, many viruses have evolved mechanisms for blocking apoptosis-including some viruses involved in cancer.

1.2.4.2 Apoptosis in pathological conditions

Both lack of apoptosis and excessive apoptosis have been implicated in various pathological conditions.

Diseases associated with inhibition of apoptosis are:-

- 1)Cancer
- 2)Autoimmune disorders
- 3)Viral infections

Diseases associated with increased apoptosis are:-

- 1)AIDS
- 2)Neurodegenerative disorders
- 3)Myelodysplastic syndromes
- 4)Ischaemic injury

Cells from a wide variety of human **cancers** have a decreased ability to undergo apoptosis in response to at least some physiologic stimuli. The role of apoptosis in cancer is discussed in detail in Section 1.2.5.

Failure to remove **autoimmune** cells that arise during development or that develop as a result of somatic mutation during an immune response can result in autoimmune disease. Recent work in animal model systems has clearly demonstrated the importance of dysregulated apoptosis in the aetiology of autoimmune disease. Fas is a cell surface receptor on activated lymphocytes stimulation of which leads to apoptosis. In mice, two forms of hereditary autoimmune disease have been attributed to alterations in Fas mediated apoptosis. MRL-lpr mice, which develop fatal systemic lupus erythematosus (SLE) by 6 months of age, have mutation in the Fas receptor. In contrast, the GLD mouse, which develops a similar illness, has a mutation in the Fas ligand. In humans a secreted form of Fas has been identified. Patients with SLE have elevated levels of soluble Fas, which may competitively inhibit Fas ligand—Fas interactions. The resultant decrease in Fas mediated apoptosis may contribute to the accumulation of autoimmune cells in this disorder. Alterations in the susceptibility of lymphocytes to die by apoptosis *in vitro* have been reported in several other diseases e.g. rheumatoid arthritis, psoriasis, inflammatory bowel disease and autoimmune diabetes mellitus (Thompson, 1995).

The disruption of cell physiology as a result of **viral infection** can cause an infected cell to undergo apoptosis. This suicide may be viewed as a cellular defence mechanism to prevent viral propagation. Cytotoxic T-cells can prevent viral spread by recognising and killing infected cells by activating the target cell's endogenous cell death programme. T-cells induce apoptosis either by activating the Fas receptor on the surface of the target cell, or by introduction of proteases, such as Granzyme B, which activate the cell death programme from within the cytoplasm. To circumvent these host defences, a number of viruses have developed mechanisms to disrupt the normal regulation of apoptosis within the infected cell. For example adenoviruses produce the E1B 19-kD protein which can block apoptosis directly. E1B has been found to be homologous to Bcl2. Poxviruses can prevent apoptosis by producing an inhibitor for the death effector molecule interleukin-1 β converting enzyme (ICE). The crmA gene of poxviruses code for a specific inhibitor for ICE. Epstein-Barr viruses up-regulate the expression of Bcl2 through the viral gene LMP-1 which is produced during latency. This provides a survival advantage to the latently infected cells.

In contrast to the above, certain viral infections, instead of preventing apoptosis, can promote apoptosis as part of their pathogenesis. The acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV) is the most dramatic example. In this condition progressive depletion of CD4 T lymphocytes leads to lymphopenia and immunodeficiency. Recent evidence suggests that stimulation of the CD4 receptor on T-cells, by its binding to the soluble viral product gp120, results in the enhanced susceptibility of these cells to undergo apoptosis (Carson and Ribeiro, 1993).

A wide variety of **neurodegenerative disorders** are characterised by the gradual loss of specific sets of neurons. Such disorders include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), spinal muscular atrophy and retinitis pigmentosa. In these diseases cell death results in specific disorders of movement and central nervous system function. In all these conditions apoptosis appears to be the main cause of neuronal cell death. The stimulus for such neuronal apoptosis has been identified in some cases. For instance, the β -amyloid plaques that accumulate in Alzheimer's disease have been shown to induce apoptosis in neurones. Free radical injury is the trigger in ALS.

Disorders of blood cell production such as **myelodysplastic syndrome** and some forms of aplastic anaemia are associated with increased apoptotic cell death within the bone marrow. These disorders could result from the activation of genes that promote apoptosis, acquired deficiencies in stromal cells or haematopoietic survival factors, or the direct effects of toxins and mediators of immune responses.

Two common diseases associated with **ischaemic injury** to cells are myocardial infarction and stroke. These diseases arise primarily as a result of an acute loss of blood flow (ischaemia). In both disorders, cells within the central area of ischaemia appear to die rapidly as a result of necrosis. However, outside the central ischaemic zone cells die over a more protracted period and morphologically appear to die by apoptosis. Agents known to be inhibitors of apoptosis *in vitro* have been shown to limit infarct size in these disorders (Solary *et al.*, 1996).

1.2.5 Apoptosis and cancer

Apoptosis plays a significant role in various stages of the natural history of a tumour from its genesis to its response to treatment.

1.2.5.1 Tumour initiation

Cancer is a clonal disease arising from a single cell which has acquired a number of genetic mutations over time. The body's response to any DNA damage is to weed out the afflicted cells by apoptotic cell death. This is achieved mainly by means of p53, a molecule that is capable of triggering apoptosis whenever it senses irreparable DNA damage (Section 1.2.3.5 b). It is because of such protective mechanisms that inspite of being constantly bombarded by carcinogens, cancer is still such a relatively rare event. If p53 function is lost, damaged cells are able to continue to divide and so any mutation arising will be fixed. That the inhibition of apoptosis through loss of p53 function is a common early event in cancer is borne out by the following facts. Aberrant p53 is the commonest genetic abnormality among human tumours, being present in as much as 50% of all cancers. In many of these tumours loss of p53 occurs early in the life history and is seen even in pre-cancerous cells. In lung cancer for instance, p53 is lost in the stage of hyperplasia and squamous metaplasia. In the Li Fraumeni syndrome, where there is inherited abnormality of p53, patients are prone to develop cancer at an early age. In mice with the p53 gene deleted, development is normal but the incidence of cancer in young animals is enormously increased (Harris, 1996).

Thus deregulation of apoptosis is likely to be a vital element in the establishment of the cancerous phenotype.

1.2.5.2 Tumour promotion

The importance of apoptosis during the stage of tumour promotion has been underscored by several experiments on rat hepatocarcinogenesis (Schulte-Hermann *et al.*, 1981; Schulte-Hermann *et al.*, 1990). There are numerous phenotypic markers which can be used to identify foci of transformed cells in rodent livers. Cell kinetic studies on these foci

reveal that DNA synthesis and mitosis are much higher within the foci than in the surrounding unaltered liver but their overall growth rate is lower than that predicted by this proliferation rate. This apparent contradiction was resolved by the discovery of a high incidence of apoptosis in altered foci which largely counterbalanced the high proliferative activity. Apoptosis, thus, functions as a defence mechanism against the development of cancer.

Tumour promoting agents such as phenobarbital were found to enhance the accumulation of preneoplastic cells in the liver foci by increasing their proliferative activity and at the same time inhibiting apoptosis. Cessation of promoter treatment resulted in massive apoptosis within the transformed foci. This may explain how tumour promotion can be reversible (Bursch *et al.*, 1984).

The tobacco alkaloids nicotine or cotinine have been shown to inhibit apoptosis in cultured cells from different origins. It has been claimed that these alkaloids might promote tumour development by inhibition of apoptosis (Wright *et al.*, 1993).

In a real life situation, the transformation of normal colorectal epithelium to carcinoma was found to be associated with a progressive inhibition of apoptosis (Bedi *et al.*, 1995). Samples were collected from normal colonic mucosa, adenomas from familial adenomatous polyposis, sporadic adenomas and frank carcinomas. These samples were disaggregated to obtain a single cell suspension and apoptotic rate was measured by serial flow cytometric analysis (at 0, 8, 16 and 24 hours) of PI stained cells. The rate of increase of the subdiploid fraction progressively diminished from normal mucosa to adenomas to carcinoma. Thus inhibition of apoptosis may contribute to tumour growth, promote neoplastic progression and confer resistance to cytotoxic anticancer agents (see Section 6.2).

1.2.5.3 Tumour progression

Studies in tissue kinetics have shown that substantial cell loss can occur even in established tumours. While some of this cell loss is accidental and necrotic, much of it is apoptotic (Kerr and Searle, 1972). There can be several triggers for apoptosis in an established tumour. In addition to the internal signal of damaged DNA (which the cell will

have had to overcome at least partially to reach the stage of frank carcinoma) there are several external factors. TNF- α has been shown to induce apoptosis in tumour cells *in vitro*. Some of the apoptosis seen in tumours *in vivo* may be attributable to the release of this cytokine by infiltrating macrophages. Apoptosis may also be the result of attack on the tumour by cytotoxic T-lymphocytes. Apoptosis is often prominent near foci of confluent necrosis where mild ischaemia is likely to be involved in its initiation. This is a known cause of apoptosis in non-neoplastic tissues. Finally, increased apoptosis in tumours may result from processes intrinsic to the tumour cells, with differing rates of apoptosis being found in otherwise similar tumours expressing different oncogenes (Kerr *et al.*, 1994).

For the tumour to expand the cells must have to be relatively refractory to such apoptotic signals so that the rate of proliferation is greater than the rate of cell loss. Such a favourable state of affairs can be achieved by alterations in one or more of the oncogenes controlling apoptosis. In most tumours the oncogenes regulating apoptosis (bcl2, myc or p53) are frequently altered indicating that deregulated apoptosis is indeed an important factor.

Bcl2 is a protein which prevents apoptosis in various types of malignant cells. In some lymphomas the rate of cell proliferation has been found to be very low (lower than in normal cells) but yet the tumour expands with time because of reduced apoptosis due to overexpressed Bcl2. The role of Bcl2 in cancer is discussed more fully in Section 1.3.6.

The acquisition of an aggressive phenotype during tumour progression is partly influenced by deregulated apoptosis. Resistance to apoptosis may select out a group of cancer cells with metastatic potential (Thompson, 1995). To maintain their viability most cells depend on environment specific signals. These may be soluble paracrine growth factors or cell-extracellular matrix (ECM) and cell-cell interactions mediated by integrin receptors on the cell surface. Withdrawal of one or more of these survival signals can result in apoptosis. This dependence may serve to prevent normal cells from surviving in non-physiological sites. Metastatic tumour cells must circumvent this homeostatic mechanism to survive at sites distant from the tissue in which they arose. Cells metastasising to serous cavities (pleura or peritoneum) are at a double disadvantage. In malignant pleural effusions for instance, the freely floating tumour cells are not only

deprived of their usual growth factors, they are also deprived of contact mediated anti-apoptotic signals. Thus cells resistant to apoptosis are more likely to survive in the circulation and colonise successfully.

1.2.5.4 Apoptosis and response to anti-cancer therapy

Both chemotherapy and radiation have had a significant impact on the treatment of many haematological and paediatric neoplasms. For adult solid tumours, like small cell lung cancer, a major impediment to successful therapy has been the failure of some tumour types to respond to either form of treatment and the appearance of resistant cell populations upon relapse of an originally responsive malignancy. The underlying basis of cellular resistance to anticancer agents has been the focus of much experimental study.

(a) Chemotherapy Numerous mechanisms of drug resistance are well established for tumour cells *in vitro*, including enhanced drug metabolism, altered drug accumulation, drug target amplification, and repair of damaged targets. Resistance to multiple drugs is often associated with overexpression of a 170 kD protein, P-glycoprotein (P-gp), the product of the *mdr1* gene. There is extensive evidence that overexpression of P-gp results in reduced accumulation of drug within the cell.

Resistance to apoptosis is a newly recognised pathway to multiple drug resistance, one that likely explains a significant proportion of treatment failures. Chemotherapy was previously thought to kill cells by inducing irreversible metabolic damages that result in target cell necrosis. It now appears that the primary mechanism by which most chemotherapeutic agents induce cell death is through creating aberrations in cellular physiology that result in induction of apoptosis. A failure of a tumour cell to engage apoptosis would therefore confer drug resistance, irrespective of the pharmacokinetic mechanisms mentioned above. Consistent with this hypothesis, over-expression of Bcl2 in different cell lines has been shown to render cells resistant to cytotoxic drugs with varying mechanisms of action. Similarly, in mouse fibroblasts, absence of p53 confers resistance to adriamycin, 5-fluorouracil and etoposide. In clinical tumours too, the presence of mutant p53 is usually associated with a poor response to chemotherapy. On the other hand,

tumours that rarely express mutant p53 at presentation e.g. testicular cancer, Wilm's tumour and childhood acute lymphoblastic leukaemia, show an extremely good response to chemotherapy (Lowe *et al.*, 1993; Harrison, 1995).

Thus, the sensitivity of tumour cells to chemotherapeutic regimens may be influenced by the expression of the apoptosis controlling oncogenes.

(b) Radiation Ionizing radiation when given in small to moderate doses greatly enhances apoptosis in tissues without producing necrosis. Rapidly proliferating cells such as stem cells and cancer cells are particularly susceptible to radiation. Among the cancer cells however, the response to radiation is variable and depends on the presence of oncogenes. The p53 gene product is directly involved in radiation induced apoptosis. When DNA is damaged by radiation p53 product accumulates within the cell and may trigger deletion of the cell by apoptosis if the damage is irreparable. Thymocytes lacking p53 are resistant to the lethal effects of radiation while retaining their normal propensity to undergo apoptosis after treatment with glucocorticoids. Tumours that are deficient in p53 are relatively radioresistant (Lowe *et al.*, 1993).

(c) Hormone Therapy Apoptosis is involved in the atrophy of endocrine-dependent organs such as the prostate, breast and adrenal cortex that follows withdrawal of trophic hormonal stimulation. Even the malignant cells that arise from these hormone dependent organs generally retain this sensitivity and undergo apoptosis causing the tumour to shrink in size. Hormone ablation has been successfully used to treat some forms of cancer e.g. the antioestrogen tamoxifen is an effective treatment for breast cancer and the antiandrogen cyproterone acetate is useful in metastatic prostatic carcinoma.

It is of great interest that Bcl2 expression may be involved in resistance to hormone therapy. Thus although Bcl2 expression was found to be virtually undetectable by immunohistochemistry in 13 of 19 cases of androgen dependent human prostate cancers, all of the androgen independent cancers studied displayed positive staining for Bcl2 protein (McDonnell *et al.*, 1992).

(d) Potential future therapeutic strategies

The selective induction of apoptosis in tumour cells offers some fascinating possibilities for treatment of cancer in the future.

A potential therapeutic strategy would be to inhibit bcl2 expression by using antisense oligonucleotides. An interesting study was performed (Smetsers *et al.*, 1994) in chronic myeloid leukaemia (CML) cells. Bcr-abl antisense oligonucleotides could specifically reduce colony formation of early haematopoietic progenitor cells from CML patients.

It has been shown *in vitro* that the treatment of tumour cells expressing Fas/Apo-1 antigen with monoclonal anti-Fas/Apo-1 antibody results in rapid selective death of these cells. This antibody could potentially be used to treat cancer (Debatin *et al.*, 1990). However, the Fas/Apo-1 antigen is also expressed on many normal cell types including peripheral blood B and T cells, liver cells and cells in the heart, lungs, thymus and ovary. When anti-Fas antibodies are injected into mice they die of fulminant hepatic failure caused by widespread apoptotic death of liver cells (Ogasawara *et al.*, 1993). To avoid complications of this nature it would be necessary to find a way to accurately target the reagents to the tumour cells.

Recently, the substitution of p53 in tumours deficient in this protein has been attempted (Roth *et al.*, 1996). A retroviral vector containing the wild-type p53 gene under control of a β -actin promoter was used to mediate transfer of wild-type p53 into human non small cell lung cancers by direct injection. Nine patients whose conventional treatments failed were entered into the study. *In situ* hybridization and DNA polymerase chain reaction showed vector-p53 sequences in post-treatment biopsies. Apoptosis was more frequent in post-treatment biopsies than pre-treatment biopsies. Tumour regression was noted in three patients, and tumour growth stabilised in three other patients.

A deeper understanding of this important physiological phenomenon can lead to many other avenues for the treatment of several human malignancies which at present evoke a dismal prognosis.

In summary it can be seen that this apparently innocuous looking cellular process (which has managed to evade the attention of biologists for well over three centuries since the invention of the first microscope) plays a vital role in many physiological and pathological processes. The biochemistry and the genetics of apoptosis is intricate and the web has not been fully untangled yet. A detailed knowledge of the genes controlling apoptosis would certainly improve our understanding of the biology of cancer. It is not possible to consider all the genes involved in apoptosis in full detail here, but, in the next two sections we shall discuss two genes - bcl2 and CD40 - as these figure prominently in our experimental work.

1.3 Bcl2

1.3.1 Introduction

Bcl2 is the acronym for the B cell lymphoma/leukaemia-2 gene. This oncogene was first discovered in 1985 by studying the t(14:18) translocation associated with human B cell malignancies like non-Hodgkin's lymphoma (Tsujiimoto *et al.*, 1985). Unlike other oncogenes which promote cell proliferation, bcl2 was found to act in a unique fashion—by preventing cell death. A large body of investigations carried out over the next few years demonstrated Bcl2's ability to prevent apoptotic cell death in a wide range of tissues. In 1993, a Bcl2 homologue called Bax was discovered which could bind with Bcl2 and prevent its anti-apoptotic action (Oltvai *et al.*, 1993). Since then several other such mutually interacting structural homologues have been identified. Some of them are death antagonists like Bcl2 (Bcl-xL, Bcl-w, Bfl-1, Bcl-1, Mcl-1 and A1) while others promote apoptosis (Bax, Bak, Bcl-xS, Bad, Bid, Bik and Hrk). This family of Bcl2 related proteins is now thought to constitute one of the biologically most relevant classes of apoptosis-regulatory gene products which play an important role in regulating cell survival in response to a variety of apoptotic stimuli including genotoxic damage, growth factor withdrawal and signal transduction through death inducing receptors such as Fas.

Normal bcl2 function is required for a variety of physiological processes. Overexpression of bcl2, by preventing the death of abnormal mutated cells, can contribute to the development of cancer. Such overexpression is frequently observed in many malignancies including lung cancer.

1.3.2 Structure

The bcl2 gene is located at chromosome 18q21 and encodes a 239 amino acid protein of 25kDa. The amino acid sequence contains no motifs that might suggest a biochemical

function for the regulation of apoptosis (Reed, 1994). Other members of the family possess variable amounts of Bcl2 homology (BH) regions (BH1 to BH4) which determine their capacity to interact with each other or with other unrelated proteins. Most members of the family including Bcl2 possess a 19 amino acid carboxy-terminal transmembrane region, but others do not (for example Bid and Bad) thereby influencing their subcellular distribution. Bcl2 is mainly located on the outer mitochondrial membrane and is also seen on the endoplasmic reticulum (ER) and nuclear membrane. Other Bcl2 family members have also been found at these locations. Bcl2's full function depends on its subcellular localisation because lack of the membrane anchor sequence partially abrogates its protective role (Kroemer, 1997).

1.3.3 Function

Vaux and associates were the first to report that bcl2 can prolong cell survival (Vaux *et al.*, 1988). Using immature Interleukin-3 (IL-3) dependent pre-B cells these investigators noticed that stable transfer of bcl2 expression vectors permitted prolonged cell survival in the absence of IL-3, but without concomitant cell proliferation. This was proved to be due to bcl2's ability to prevent apoptosis. On the other hand, antisense mediated reductions in bcl2 gene expression were shown to accelerate the rate of cell death in settings of growth factor withdrawal (Reed *et al.*, 1990).

Bcl2 is now known to be capable of preventing apoptosis due to a wide range of stimuli in a wide range of cell types with a few exceptions, the most notable being apoptosis during cytotoxic T-cell mediated killing (Vaux *et al.*, 1992). This general inclusiveness of bcl2's anti-apoptotic function indicates that it must act after the convergence of many signals in the apoptotic pathway. Indirectly, this has led to the expectation that a final common pathway operates in all forms of apoptosis and furthermore, that elucidation of the molecular function of bcl2 is a key to understanding this mechanism.

There are many similarities between the apoptotic mechanisms observed in humans and other lower forms of life like the nematode *C. elegans* (see Section 1.2.3.2). The *C.*

elegans gene *ced-9* prevents apoptosis by inhibiting the action of two other genes *ced-3* and *ced-4*. *Ced-9* is structurally and functionally similar to *Bcl2*; so much so that human *bcl2* can inhibit cell death in nematodes and can even partially substitute for the loss of *ced-9* function. *Bcl2* homologues have been found in many other species and in rodents such homologues bear a 70-80% structural similarity with the human protein.

Thus from worms to lower mammals to humans, *bcl2* seems to have been conserved in the genetic machinery of all multicellular organisms over hundreds of millions of years of evolution and must therefore serve some important purpose. Broadly, it appears that the death repressor activity of *bcl2* plays a crucial role in two important physiological situations.

- 1) Embryonic development;
- 2) Control of cell numbers in tissues.

1.3.3.1 Embryonic development

Normal embryonic development requires the selective apoptotic death of groups of cells. The *Bcl2* group of proteins are widely involved in the regulation of such developmental apoptosis. Experiments on gene disruption animal models have shown that there are clear differences in the lineage specificity of the various family members.

During embryogenesis, *bcl2* is initially widely expressed but later its distribution becomes confined to the developing kidney, lymphoid organs and skin, where its presence is essential for normal development. *bcl2* ^{-/-} knockout mice are normal at birth but then become ill and die of renal failure within a few weeks. They develop polycystic kidney disease due to greatly increased apoptosis within the developing kidney. The lymphoid organs undergo atrophy due to massive cell death. Decreased melanocyte survival results in hypopigmentation (Veis *et al.*, 1993).

While the absence of *bcl2* allows viable mice to be born, the absence of *bcl-x* results in embryonic lethality. In contrast to *bcl2*, *bcl-x* appears to be essential for brain development and in *bcl-x* ^{-/-} embryos there is extensive cell death throughout the brain and spinal cord. Massive cell death is also seen in the liver. (Motoyama *et al.*, 1995)

bax^{-/-} mice are born healthy but later develop lymphoid hyperplasia and females show excess granulosa cells in atretic ovarian follicles consistent with excess cell survival. However, males are infertile and show massive apoptosis of germ cell precursors (Knudson *et al.*, 1995).

Thus not only is there lineage specificity in the bcl2 family members, but depending on the cell type the same molecule can have a positive or a negative effect on cell death.

1.3.3.2 Control of cell numbers in tissues

bcl2 is less widespread in adult than in foetal tissues. It is seen in lymphoid, haematopoietic, epithelial and neural tissues where its expression is limited to distinct cell types or topological areas. By such differential expression bcl2 helps to protect cells which are vitally important. For instance, in lymphoid organs differential bcl2 expression helps to protect memory B cells and plasma cells.

bcl2 is normally expressed in a wide variety of epithelia including bronchial epithelium, gastro-intestinal epithelium, thyroid, breast, prostate and skin (Hockenberry *et al.*, 1991). In normal bronchial epithelium, only cells in the basal layers express bcl2 but the vast majority of differentiated cells are negative (Walker *et al.*, 1995). The basal areas of bronchial epithelium contains stem cells and other undifferentiated cells which must be kept alive for their regenerative powers. As the cells differentiate they move up into the superficial layers and eventually undergo apoptosis and are shed into the bronchial lumen to make way for new cells. In a sense apoptosis can be considered to be a form of terminal differentiation. If for any reason bcl2 persists in these differentiated cells they will accumulate in large numbers and may acquire new mutations which can lead to cancer.

Hockenberry has suggested that in certain tissues, in addition to its well documented role in preventing apoptosis, bcl2 might have some other biological functions as well (Hockenberry, 1995). In certain locations, such as the germinal centres of lymph nodes, the anti-apoptotic role of bcl2 is self evident. In other situations, cells which express bcl2 are not known to require enhanced survival functions e.g. the mesenchymal elements associated with respiratory epithelium. It is speculated that at these sites bcl2 could play

some role in cell differentiation and proliferation. BHRF1, a viral homologue of Bcl2 (see Section 1.3.4 below), when transfected into the human squamous cell carcinoma cell line SCC12F was found to delay the terminal differentiation of these cells (Dawson *et al.*, 1995). It is certainly not unknown for genes to be involved in more than one cellular process e.g. c-myc is involved in both cell proliferation and cell death. Additional support for this added role for bcl2 has come from some observations in the kidneys of bcl2 ^{-/-} mice. As mentioned above, they develop polycystic kidney disease, but the histological picture is heterogeneous. In addition to marked apoptosis in the mesenchymal cells but there is also hyperproliferation in interstitial and glomerular epithelial compartments. Whether the latter is a direct effect of lack of bcl2 is not known yet. New experimental evidence has emerged which indicates that bcl2 can retard the rate of cell proliferation in cultures (Borner, 1996).

1.3.4 The Bcl2 family

The functions of Bcl2 are modulated by interactions with other proteins which bind to it. Most of these proteins are structurally very similar to Bcl2 but they have opposite effects on cell death. Some of them are death antagonists while others are agonists. In general, the numerical ratio of these agonists and antagonists within a cell determines its response to an apoptotic signal. This ratio probably depends on cell lineage and state of differentiation but it can be modulated by other genes. p53, for instance, can upregulate the transcription of bax and downregulate bcl2, thus altering the ratio in favour of apoptosis. However, there are situations where mere numerical superiority is not enough. Ras, by phosphorylating Bcl2 can prevent it from binding to other proteins and thus reduce its effectiveness. Also, mutations in the BH1 and BH2 regions of Bcl2 have been induced in the laboratory, which can alter its ability to bind to other proteins (Yin *et al.*, 1994). Whether such mutations take place spontaneously in tumours is not known yet.

Bax, was the first protein identified by its co-immunoprecipitation with Bcl2. It counters the survival enhancing effects of bcl2 in co-transfection experiments, proportional to the

Bax/Bcl2 ratio. Bcl2 and Bax interact as homo- and heterodimers. The opposite cellular functions demonstrated for Bcl2 and Bax have raised the question of whether one dimer partner acts as a dominant negative inhibitor of the other partner's function. bax is widely distributed in tissues including a number of sites in which cells die during maturation.

The bcl-x gene was isolated through its sequence similarity to the bcl2 gene. Bcl-x_L (large), the larger protein product of the human bcl-x gene, resembles Bcl2 in its ability to inhibit apoptosis in growth factor deprived cell cultures but the two proteins are expressed in different types of cells and perhaps fulfil different physiological roles. Bcl-x_S (short) counters the protective effects of both Bcl2 and Bcl-x_L.

Bak (*Bcl2* homologous antagonist killer) is functionally similar to Bax. It interacts with Bcl2 and Bcl-x_L and opposes their death repressor activity when co-expressed in cultured cells. Bak differs from Bax in its preference for heterodimerizing partners. Bak appears to prefer Bcl-x_L over Bcl2. Bak and Bax may also have different cell type specificities as has been shown for Bcl2 and Bcl-x_L.

Yeast two hybrid screening revealed a new heterodimerizing partner of Bcl2 and Bcl-x_L called Bad (*Bcl2/Bcl-x_L associated death promoter*) which is found only in murine tissue. Like Bax, Bak and Bcl-x_S, Bad promotes apoptosis in cell cultures. Bad lacks the membrane anchor region characteristic of other family members.

In conclusion, it appears that members of the Bcl2 family interact with each other to form a dynamic equilibrium between homo- and heterodimers. Current data do not clarify which dimers are true regulators of apoptosis. (For review of bcl2 family members:- Yang and Korsmeyer, 1996; Kroemer, 1997).

Two other bcl2 like genes were initially found to be expressed in specific cell lines in response to specific stimuli. Mcl-1, which was cloned from a myeloid leukaemia line after induction by phorbol ester, is a negative regulator of apoptosis. Its pattern of expression differs from those of Bcl2 and Bcl-x_L. It is expressed in epithelial cells that are more differentiated than those expressing Bcl2 and in various types of muscle and neuroendocrine cells (Kozopas *et al.*, 1993). A1 is induced in several murine haematopoietic cell lines by GM-CSF and lipopolysaccharide (LPS). In spite of its

structural similarity with bcl2 the precise role of this gene in cell death is still not clear (Lin *et al.*, 1993).

Some viruses display genes which have functional and/or structural similarities with bcl2. The apoptosis inhibiting adenovirus E1B 19-kDa protein shows only minimal amino acid sequence similarity to Bcl2 but functionally the two are very similar and Bcl2 can substitute for E1B in preventing apoptosis in adenoviruses. Other viral genes which encode Bcl2 related proteins include the Epstein-Barr virus BHRF1 gene (Pearson *et al.*, 1987); the African swine fever virus (AFSV) LMW5-HL gene; the herpesvirus saimiri ORF16 gene. It is likely that these proteins prevent or delay the apoptosis of infected cells to prolong viral replication.

1.3.5 Mechanisms of action

Although much is known about the interaction between the various Bcl2 family members, the actual mechanism by which bcl2 prevents apoptosis still remains shrouded in mystery. Several conflicting theories have been proposed but none has yet been proved to be true.

(a) From the present evidence, the likeliest explanation of Bcl2's prowess lies in its ability to interact with the Ced-3/ICE-like proteases. These enzymes when activated lead to downstream events in the nucleus and cytoplasm which culminate with the cell dying by apoptosis. Bcl2 is able to prevent the Ced-3/ICE-like proteases from having this effect but there is no experimental evidence of direct chemical interaction between the two types of molecules which might suggest how this happens. Bcl2 certainly does not control the expression of the Ced-3/ICE-like proteases for it is known to be effective even in enucleated cells (see Section 1.2.3.5 a). It now seems that Bcl2 modulates Ced-3/ICE function indirectly through its effect on Ced-4. Recently a human homologue of the nematode Ced-4 has been identified and named Apaf-1 (apoptotic protease activating factor-1) (Zou *et al.*, 1997). Apaf-1 is an adaptor protein that can receive an apoptotic signal, bind to pro-Ced-3 and activate it. By binding to Apaf-1, Bcl2 somehow prevents it from activating pro-Ced-3.

There is now evidence that the Ced-3/ICE enzymes might not be involved in every form of apoptosis and Bax and Bak overexpression have been found to induce death in the presence of Ced-3/ICE inhibitors (McCarthy *et al.*, 1997).

(b) Many of the changes of apoptosis are thought to be due to excess production of reactive oxygen species (ROS) like peroxide or superoxide O_2^- . Although Bcl2 cannot prevent the production of ROS it can protect the cell from their ill effects by inhibiting lipid peroxidation, a downstream event in oxidation (Hockenberry *et al.*, 1993). However, Bcl2 does not lose its protective power even in hypoxic conditions, in which the generation of ROS is greatly reduced, indicating that its anti-oxidant effect may not be the sole explanation of its ability to prevent cell death.

(c) Apoptosis is associated with an efflux of Ca^{2+} from the endoplasmic reticulum (ER) to the cytosol and Bcl2 can block this flux across the ER membrane. It is not clear if these alterations in Ca^{2+} contribute functionally to the apoptotic process because some cases Bcl2 is able to block apoptosis despite a rise in cytosolic calcium concentration (Lam *et al.*, 1994).

(d) It has been suggested that Bcl2 modulates the transport of ions and proteins across biological membranes to which it is attached i.e. mitochondrial, ER and nuclear membranes. Both Bcl2 and Bcl-x_L contain an amino acid sequence motif which is present in some nuclear pore proteins and deletion of this motif reduces the anti-apoptotic activity of Bcl2. The three dimensional structure of Bcl-x_L was also shown to contain an element similar to the pore forming domain of bacterial toxins. Like these bacterial toxins, Bcl-x_L is able to insert ion conducting channels into biological membranes. It is possible that through these channels Bcl-x_L controls the intracellular distribution of a molecule that is crucial in apoptosis. One likely candidate is cytochrome c which normally resides in mitochondria but once in the cytoplasm it along with Apaf-1 is essential for the activation of ICE/Ced-3 like proteases (Minn *et al.*, 1997; Vaux, 1997). It has been hypothesised that the key function of Bcl2 is to somehow retain cytochrome c in the mitochondria.

Thus Bcl2 seems to be a versatile protein with multiple independent functions rather like a Swiss army knife. It can function both as a channel protein and as an adaptor/docking protein to achieve the same end i.e inhibition of ICE/Ced-3 (Reed, 1997).

1.3.6 Bcl2 and cancer

Although it has subsequently been found to be involved in many other physiological and pathological conditions it was bcl2's involvement in malignancy that first sparked interest in this gene.

In 85% of follicular lymphomas bcl2 is translocated from its normal position in 18q21 to position 14q32. This t(14:18) translocation places the gene in juxtaposition to powerful elements in the immunoglobulin heavy chain locus (IgH) resulting in overproduction of Bcl2 protein which is normal in every other respect (Brada, 1992). To assess the contribution of this translocation and overexpression of bcl2 to neoplasia a series of experiments were devised.

In normal haemopoietic cell lines *in vitro*, overproduction of Bcl2 by transfection of the gene did not immortalise them or induce proliferation but prolonged cell survival by inhibiting apoptosis. In the same cell lines, when c-myc was activated in addition, it resulted in profuse cell proliferation which was growth factor independent (Fanidi *et al.*, 1992). There were similar findings from parallel experiments on transgenic mice with deregulated bcl2 gene. These mice initially developed follicular lymphoid hyperplasia involving the spleen, lymph nodes and bone marrow. After a latency period of about 15 months some of these mice progressed to malignant lymphoma and this transformation was usually associated with the activation of the c-myc oncogene. Thus bcl2 alone is incapable of causing malignancy and its main role seems to be to keep the cells alive for long enough to acquire a second oncogenic hit. Often the second hit results in a deleterious mutation which normally would have resulted in apoptotic cell death were it not for the presence of excess Bcl2. Instead the abnormally mutated cells are allowed to thrive and cause cancer (Marin *et al.*, 1995). These experimental findings are compatible with the clinical experience of the behaviour of low grade lymphoma. The disease is characterised by a long indolent phase [t(14:18) bcl2 deregulation] followed by a highly malignant phase (c-myc activation).

Bcl2 overexpression has now been documented in a wide range of other malignancies of both lymphoid and epithelial origin. As in lymphomas, in many carcinomas too bcl2

overexpression has been shown to take place from an early pre-malignant stage. In lung cancer for instance, *bcl2* overexpression is evident even in dysplastic epithelium where one of the earliest changes is alteration of the spatial distribution of Bcl2. Instead of being confined to the basal layers, *bcl2* positive cells are also seen in the more superficial layers of dysplastic bronchial epithelium. With increasing severity of dysplasia, the staining becomes more prominent with a greater proportion of *bcl2* positive cells in the upper layers (Walker *et al.*, 1995). Similar changes have also been documented in pre-malignant lesions of the stomach and colon. It seems very likely that as in lymphomas in carcinomas too, *bcl2* may help in tumour promotion by prolonging the life-cycle of the cells and making them more tolerant towards new mutations.

Unlike lymphomas where the t(14:18) translocation is responsible, in cancers the molecular mechanism underlying *bcl2* overexpression is not known yet. No evidence of translocation or amplification of the *bcl2* gene has been found in any cancer and it is thought that post-translational mechanisms are involved. The finding that the protein is absent or weakly expressed in proliferating lymphocytes, although *bcl2* messenger RNA increases in peripheral blood lymphocytes after *in vitro* stimulation, suggests post-transcriptional regulation as a possible physiologic mechanism controlling the expression of *bcl2* (Reed *et al.*, 1987). If this is so, alterations in the control of messenger RNA translation could lead to aberrant accumulation of *bcl2* protein.

Whatever the cause of the overexpression it certainly persists in a sizeable fraction of all cancers till a late stage and probably contributes to tumour progression. The biological role of *bcl2* at these late stages has not been defined clearly and it seems to vary widely according to the site and type of tumour (see Section 6.7).

The overexpression of *bcl2* can result in diametrically opposite clinical consequences in different tumours. In lymphoma, acute myeloid leukaemia and adenocarcinoma of prostate elevated *bcl2* is associated with poor response to chemotherapy and radiation and with shorter survival. This is easy to understand from the known biological effects of *bcl2*. Chemotherapy, radiotherapy and hormone ablation all kill tumour cells by apoptosis. Therefore cells which have excessive *bcl2* would naturally be resistant to these forms of treatment. By preventing apoptotic death, *bcl2* would lead to rapid expansion of tumour

mass and poor prognosis. Far less intuitive is the effect that bcl2 is seen to produce in some other tumours e.g. breast cancer and lung cancer. In both these tumours bcl2 seems to be able to prolong survival and in breast cancer, presence of bcl2 is paradoxically associated with a better response to chemotherapy and hormone therapy. Why this should be is not clear. It seems that oncogenes may have distinct biological effects depending on the cellular context and the presence of other factors e.g. the co-expression of other oncogenes.

In the next section we shall discuss another apoptosis controlling gene (CD40) whose existence has been known for almost as long as that of bcl2. But unlike bcl2 the role of this gene in apoptosis (particularly in epithelial cells) has not been fully defined yet.

1.4 CD40

1.4.1 Introduction

In 1984, while investigating Tumour Associated Antigens (TAAs), Hannu Koho and colleagues isolated a monoclonal antibody, S2C6, by immunizing mice with urinary bladder cancer cells. They showed that this antibody reacted with transitional cell carcinoma of bladder while sparing normal bladder epithelium. They also showed that cells of B-lymphocyte origin expressed the S2C6 antigen (Koho *et al.*, 1984). By 1989 it was clear that this antigen was an important B-cell surface molecule and it was renamed CD40 at the Fourth International Workshop on Leukocyte Antigens in Vienna.

CD40 belongs to a growing family of molecules including Low affinity Nerve Growth Factor-Receptor (LNGFR), Tumour Necrosis Factor-Receptor (TNFR) and Fas which are involved in the transmission of apoptotic/anti-apoptotic signals. Collectively they comprise the so called TNFR superfamily. CD40 is an activation molecule for B-cells since its cross linking by CD40 ligand (CD40-L) augments proliferation of resting B-cells, promotes immunoglobulin isotype switching and prevents apoptosis in germinal centre B-cells. CD40-L is expressed on activated T-cells and the CD40/CD40-L interaction plays a key role in T-cell dependent B-cell activation.

In addition to lymphocytes CD40 is also expressed on various epithelial cells and on carcinomas at various sites. It is speculated that CD40 might have a role in the genesis of some of these neoplasms.

1.4.2 Distribution

CD40 is a pan B cell antigen. It is acquired early in B cell ontogeny, retained through the different stages of B cell development and finally lost during terminal B cell differentiation. Thus, plasmablasts express CD40 but plasma cells do not. It is expressed on malignant B

lymphocytes as well. Cells from B lineage acute lymphoblastic leukaemias (B-ALL), B-chronic lymphocytic leukaemias (B-CLL), Non Hodgkin's Lymphoma (NHL) and the great majority of multiple myeloma cells express CD40. Reed Sternberg cells, the malignant cells of Hodgkin's Disease which are purportedly of B cell origin, express CD40. (Banchereau *et al.*, 1994)

T lymphocytes, in contrast, do not express CD40 but some of them express the ligand CD40-L (see below). Within lymphoid organs CD40 is also expressed on antigen presenting cells (APCs) like interdigitating dendritic cells, follicular dendritic cells and monocytes.

CD40 was initially identified as an antigen on urinary bladder cancer cells. It has now been found on epithelial cells at various other sites - both on normal and neoplastic epithelium. Concerning normal epithelium, CD40 is expressed on the basal (proliferating) epithelial layer of nasopharynx, tonsil, ectocervix and on gastrointestinal and bronchial mucosa (Young *et al.*, 1989). This preference for the basal cells is highly reminiscent of the distribution of *bcl2* in normal epithelium (see Section 1.3.3.2). It is also found on thymic epithelial cells and on cultured proximal tubular epithelial cells (PTEC) in the kidney. CD40 antigen has been identified on carcinomas at various sites such as colon, prostate, breast and lung as well as on skin melanomas (Ledbetter *et al.*, 1987; Stamenkovic *et al.*, 1989).

CD40-L is expressed on activated mature T-cells but not on resting T-cells. It is primarily restricted to CD4⁺ cells. On immunohistological analysis such CD40-L expressing cells are mainly found in the germinal centres of lymphoid follicles. This is the site where CD40 dependent apoptotic rescue of B-cells takes place. (see Section 1.4.4.1)

The expression of CD40-L on activated T-cells is transient and seems to be tightly regulated. On activation of cultured CD4⁺ T-cells *in vitro*, CD40-L is expressed after 1-2 hours and reaches a maximum at 6-8 hours before gradually declining. In addition to this intrinsic transient expression, *in vivo* the close proximity of B-cells further limits prolonged CD40-L expression and thus prevents protracted CD40/CD40-L interactions. Following receptor ligand binding there is (a) downregulation of CD40 mRNA and (b)

receptor mediated endocytosis of CD40-L followed by its lysosomal degradation. Also B-cells are capable of releasing soluble CD40 (sCD40) which can bind to CD40-L and in effect inactivate it. (van Kooten *et al.*, 1994; Graf *et al.*, 1995)

Lung and skin mast cells and blood basophils also express CD40-L.

1.4.3 Structure

The CD40 antigen is a phosphorylated glycoprotein of 48kDa. It has 277 amino acids with a 193 amino acid extracellular domain, a 22 amino acid transmembrane domain and a 62 amino acid intracellular tail. The extracellular segment is similar to other TNFR proteins in its organisation. It has four protein domains of 40 amino acids with six cysteine residues each, which is the hallmark of this family of cell surface receptors. The intracellular tail of CD40 is quite unique and bears no relationship to those of other family members suggesting that it has different signalling properties. It has a Threonine residue at position 234 which is critical for signal transduction (see Section 1.4.5.2).

In addition to the membrane bound form, B cells are also capable of releasing a soluble form of CD40 - sCD40 - which might have important regulatory roles. The soluble receptor is created either by proteolytic cleavage of the transmembrane protein or by alternative splicing of CD40 mRNA.

The gene coding CD40 antigen is located on human chromosome 20q11 - 20q13-2.

CD40-L is a transmembrane protein of 261 amino acids with a 22 amino acid intracellular domain, a 24 amino acid transmembrane domain and a 215 amino acid extracellular domain with five cysteines. A major portion of the extracellular domain (~200 amino acids) shows a strong similarity to other TNF-like molecules. This region comprises the bio-active, receptor binding, globular portion of CD40-L which assumes a "β-jellyroll" topology characteristic of all TNF family members.

The gene coding for CD40-L is located on human chromosome X-q24.

(For a detailed review of CD40 and CD40-L structure *vis a vis* other TNF Superfamily members:- van Kooten and Banchereau, 1996)

1.4.4 Function

The function of CD40 will be discussed in the context of the three main types of cells in which it is known to be expressed.

1.4.4.1 B Lymphocytes

CD40 is one of the key molecules involved in the survival, growth and differentiation of B lymphocytes.

Within the B lymphocytes the elimination of cells reactive with cross linking self antigens occurs in the bone marrow (where it may or may not involve apoptosis). Further censoring of the mature B cell repertoire for autoreactivity occurs within the germinal centres of the peripheral lymphoid organs following the induction of high rate somatic mutation in Ig V region genes. Here germinal centre B cells apparently enter an apoptosis prone stage in their differentiation. Non self-reactive B cells can be rescued from apoptosis by the activation of CD40 which causes upregulation of bcl-x_L (see Section 1.4.5.3). The stimulus for activation is provided by the nearby CD4⁺ T cells which express CD40-L on their surface. Thus, CD40/CD40-L is at the very heart of this vital T-B cell interaction which shapes the immune response (vanKooten and Banchereau, 1996).

B cells which have been thus protected from apoptosis by CD40 also show signs of activation as evidenced by increased size, tendency to form homotypic aggregates and increased adhesiveness to endothelial cells. If these cells are co-stimulated with cytokines like IL-4 and IL-10 they will proliferate rapidly. Indeed B cells have been shown to produce various cytokines e.g. IL-1 β , IL-6, IL-10, TNF α , LT α and GM-CSF in response to CD40 activation (Burdin *et al.*, 1995). These may serve as autocrine growth and differentiation factors.

The engagement of CD40 on B cells seems to turn on the immunoglobulin isotype switching machinery, the specificity of which is subsequently provided by cytokines. For example, IL-4 and IL-13 lead to increased IgE whereas IL-10 and TGF- β induce IgA production. CD40/CD40-L interactions also appear to play an important role in the subsequent development of B cells. *In vitro* studies have shown that proliferating centrocytes will mature into memory B cells in response to prolonged CD40 triggering and into plasma blasts when CD40 signalling is limited and IL-10 is available (Arpin *et al.*, 1995).

Although CD40 protects B cells from apoptosis induced by surface immunoglobulin crosslinking (which is the main apoptotic stimulus within germinal centres) its role in Fas-induced apoptosis is exactly the opposite. CD40 activation has been shown to upregulate the expression of Fas antigen and increase the susceptibility to Fas mediated apoptosis in murine B cells (Koizumi *et al.*, 1996) as well as normal and malignant human B cells (Schattner *et al.*, 1995; Wang *et al.*, 1997).

The importance of the CD40 signalling system in immune regulation is underscored by the Hyper IgM (HIGM) syndrome. In this X-linked disorder, the gene encoding CD40-L is mutated or deleted (Callard *et al.*, 1993). Males affected by the HIGM syndrome are susceptible to bacterial infections. They do not make antibodies to exogenous antigens but make a variety of auto-antibodies. Their serum has vastly elevated concentrations of IgM and IgD but no detectable IgA or IgE and very low levels of IgG. The secondary lymphoid organs of these patients display no germinal centres.

1.4.4.2 Antigen presenting cells

On antigen presenting cells the effect of CD40 activation is in general stimulatory leading to increased growth, cytokine secretion and other signs of activation. In contrast to B cells, in dendritic cells CD40 ligation induces resistance to Fas induced apoptosis by a mechanism which involves upregulation of bcl2 rather than bcl- χ_L (Bjorck *et al.*, 1997).

1.4.4.3 Epithelial cells

The physiological role of CD40 may be broader than initially suggested by its pan B cell expression. As stated before (Section 4.2) CD40 is expressed on a wide range of normal and malignant epithelium. Although its exact biological role in these cells has not been defined fully yet, even the early studies had indicated that CD40 might be a functional molecule on epithelial cells. Thymic epithelial cells are induced to secrete GM-CSF following triggering with soluble anti-CD40 in conjunction with IL-1 or IFN- γ , the latter upregulating CD40 expression (Galy and Spits, 1992). This could have implications for the growth of both normal and transformed epithelial cells.

There are conflicting reports about the effects of CD40 ligation on the growth of epithelial cells. Unlike its protective role in B cells CD40 ligation on many epithelial cells has been shown to induce apoptosis. In both normal primary epithelial cells as well as in carcinoma cell lines, CD40 ligation resulted in growth inhibition and enhanced susceptibility to apoptosis induced by antineoplastic drugs, TNF- α , Fas and ceramide. Interestingly, the expression of Bcl2 did not affect the growth inhibition induced by CD40 ligation in these cells (Eliopoulos *et al.*, 1996). Deglieposti *et al.* found CD40-L to cause apoptosis of bladder carcinoma cell lines T24 and RT4, while Hess and Engelmann found the same effect on many types of transformed cells of epithelial and mesenchymal origin (Deglieposti *et al.*, 1995; Hess and Engelmann, 1996).

In sharp contrast, in prostatic cancer cell lines, CD40 was found to protect the cells from Fas and TNF- α mediated apoptosis (Rokhlin *et al.*, 1997). Further detailed investigations are clearly necessary to clarify the situation. It may be that as in B cells, in epithelial cells too, CD40 may have pro and anti apoptotic roles in different situations in different cells.

1.4.5 Signal transduction through CD40

Studies indicate that CD40 ligation activates protein tyrosine kinases (PTKs) including Lyn and Syk and results in tyrosine phosphorylation of multiple substrates including

phosphatidyl inositol 3-kinase and phospholipase C- γ 2. However the cytoplasmic segment of CD40 does not have any intrinsic enzymatic activity and signal transduction is likely to be mediated by associated molecules. There is evidence for at least three possible pathways of signal transduction:-

- 1) CRAF-1;
- 2) NF- κ B;
- 3) Bcl2/Bcl-x_L.

1.4.5.1 CRAF-1

TNF receptors in general have been found to require the help of a group of proteins called TRAF (TNF receptor associated factors) to transmit signals. Recently, using the two hybrid system, a similar protein has been found which specifically assists in CD40 signal transduction. It has been named CRAF-1 (CD40 receptor associated factor 1). This 567 amino acid protein has several important functional domains.

CRAF-1 has a string of Zn fingers that can bind to DNA. Close to the NH₂ terminal it also has a Zn ring structure which, in other proteins, has been shown to have diverse DNA related functions including recombination, repair and transcription regulation. These structural data suggest that after activation CRAF-1 could dissociate from the cytoplasmic tail of CD40 and directly transmit transcriptional signals to the nucleus. The CRAF-C domain at the carboxy terminal helps to bind CRAF-1 to the cytoplasmic tail of CD40. Its presence is also essential for CRAF-1 to form homo- and/or hetero-oligomers with other members of the TRAF family which is thought to occur following ligand binding. CRAF-1 mediates an anti-apoptotic signal while the other TRAF members transmit a pro-apoptotic signal. It is possible that apoptosis and cell survival may be determined by an equilibrium of dimerization between TRAF family members (Cheng *et al.*, 1995).

1.4.5.2 NF- κ B

NF- κ B is a transcription factor that is utilised by several members of the TNFR family. Activation of CD40 induces the transcription factor NF- κ B by a mechanism which seems to require the T234 residue. NF- κ B binds to κ B sites within the A20 promoter gene. This

results in increased production of A20 within an hour of CD40 activation. A20 is a novel zinc finger protein that confers at least partial resistance against apoptosis. It seems probable that the heightened state of resistance to apoptosis conferred by CD40 activation is due to synergistic interaction between A20 and a Bcl2 like protein e.g. Bcl-X_L which is also CD40 inducible (Sarma *et al.*, 1995).

1.4.5.3 Bcl2/Bcl-x_L

The relation between CD40 and the Bcl2 family of proteins is controversial and has been the subject of considerable debate. Given the protective effects of Bcl2 against apoptosis in several models, it was speculated that the anti-apoptotic effects of CD40 activation might be implemented through Bcl2. In fact early evidence did seem to point to this fact. Kamesaki *et al.* demonstrated in 1994 that Bcl2 appears to confer partial protection against anti-IgM induced apoptosis in the immature B cell line CH31 (Kamesaki *et al.*, 1994).

However, subsequently it has emerged that CD40 mediated apoptotic rescue of B cells is probably independent of Bcl2 but needs the co-operation of Bcl-x_L, which is a member of the Bcl2 family (see Section 1.3.4). Most of this work has been done on WEHI-231 a murine B lymphoma cell line that resembles immature B cells. Wang *et al.* showed that Bcl-x_L is upregulated within three hours of CD40 engagement and protects WEHI-231 cells from sIg induced apoptosis. Antisense oligonucleotides to Bcl-x_L can partially block this CD40 mediated apoptotic rescue. In contrast the expression of Bcl2 is not significantly affected by CD40 activation (Wang *et al.*, 1995). Further, studies on Bcl2 deficient mice showed that CD40 ligation can prevent B cell apoptosis even in Bcl2 negative cells (Nakayama *et al.*, 1995). Several other investigators have reported that bcl-x_L plays an important role in CD40 mediated rescue of B cells (Ishida *et al.*, 1995; Tuscano *et al.*, 1996; Fang *et al.*, 1997).

In contrast, in their experiments on the B cell lines CH31 and WEHI-279, Koizumi *et al.* found no evidence of upregulation of bcl-x_L following CD40 ligation (Koizumi *et al.*, 1996). Again, in human dendritic cells, Bjorck *et al.* have shown that the ligation of CD40 results in the upregulation of Bcl2 protein expression (Bjorck *et al.*, 1997)..

Thus it seems that CD40 recruits different bcl2 family members in different cell types. There is as yet no information about which, if any, bcl2 family member is involved in CD40 mediated signal transduction in epithelial cells. Further studies are necessary.

1.4.6 CD40 and carcinogenesis

Ever since CD40 (the erstwhile S2C6 antigen) was identified on urinary bladder cancer cells in 1984 there has been speculation that it might, in some way, be linked to the genesis of epithelial neoplasms. The initial observation (now proved wrong) that it was selectively expressed on cancer cells while sparing normal epithelium, added fuel to this view. If CD40's demonstrated proliferative and anti-apoptotic influence on B lymphocytes can be proved to be true in the context of epithelial cells as well, one could easily imagine a role for it as an oncogene in carcinomas. Meanwhile the discovery of CD40 on an ever increasing number of carcinomas at diverse sites continues to provoke research in this area.

1.4.6.1 Lymphoid tumours

There are good grounds to believe that CD40 is involved in the pathogenesis of some tumours of lymphoid origin e.g. Hodgkin's disease and multiple myeloma.

The malignant cells in Hodgkin's disease (HD) are the Hodgkin and Reed Sternberg cells (HRS). Although the origin of these cells is still unclear (could be T cells, B cells or APCs) there is evidence that dysregulation of CD40 in these cells may play a role in the pathogenesis of HD. Overexpression of CD40 compared to normal B cells has been found on most HD cases studied immunohistologically (Ogrady *et al.*, 1994) and on all HD derived cell lines examined flow cytometrically for surface expression and by Northern blot analysis for mRNA expression. In all HD derived cell lines, binding with CD40-L led the cells to produce cytokines like IL-6, IL-8, TNF and LT- α and induced the expression of cell surface molecules like ICAM-1 and B7-1. Despite this, CD40 crosslinking has not been demonstrated to have any mitogenic effect on these HD derived cell lines. This could

be due to *in vitro* loss of response to CD40-L or on the need for additional co-stimulatory compounds such as cytokines or cellular antigens. The model of HD caused by CD40 dysregulation still remains attractive. One intriguing possibility is that the abundant non-malignant T cell infiltration seen in HD may provide the proliferative stimulus through the CD40-L expressed on their surface (Gruss *et al.*, 1994). T cell mediated CD40 signalling is also thought to be essential for the development and progression of low grade MALT-type (MALT = mucosa associated lymphoid tissue) B cell lymphomas (Greiner *et al.*, 1997).

In contrast, in various aggressive histology B cell lymphomas and cell lines, both *in vivo* and *in vitro*, CD40 stimulation can inhibit growth (Funakoshi *et al.*, 1995). This indicates that CD40 stimulation is selective in its effects among B cells.

CD40 expression in multiple myeloma cells may play an important role in tumour cell expansion by inducing autocrine IL-6 secretion. IL-6 serves as a primary growth factor for myeloma cells both *in vivo* and in culture *in vitro*. It has been shown that CD40 stimulation leads to cell proliferation and to increased IL-6 mRNA levels in ANBL-6 cells derived from multiple myeloma (Tong *et al.*, 1994).

1.4.6.2 Epithelial tumours

Information about the role of CD40 in epithelial tumours is scarce and whatever little is available is often contradictory.

In 1989 Stamenkovic *et al.* showed that IFN- γ upregulated the expression of CD40 mRNA in carcinoma cell lines HS294T (melanoma) and HepG2 (hepatocarcinoma). Since IFN- γ is produced by activated T cells at sites of inflammation and since cancers often arise at sites of chronic infection and inflammation (e.g. hepatocellular carcinoma associated with chronic active hepatitis and cervical squamous cell carcinoma arising in the context of herpes virus infection) it was reasoned that CD40 activation by infiltrating T cells might have a role in carcinogenesis at these sites (Stamenkovic *et al.*, 1989)

Agathangelou *et al.*, investigating undifferentiated nasopharyngeal carcinomas (UNPC) found CD40 expression in virtually all tumour cells. UNPCs are characterised by the presence of an intense lymphoid stroma in most cases. A large number of these cells are

CD40-L positive T lymphocytes. The authors speculated that these infiltrating T cells might promote tumour cell growth through CD40/CD40-L interactions (Agathangelou *et al.*, 1995). This is rather reminiscent of the scenario in Hodgkin's Disease. The CD40/CD40-L growth loop seems to be important in the progression of malignant melanomas (MM) as well. 71 cases of MM were investigated immunohistologically for the expression of CD40 in tumour cells and CD40-L in infiltrating cells. Tumours that were CD40+/CD40-L+ behaved more aggressively and had a significantly shorter tumour free survival period (Vanderoord *et al.*, 1996). In this context one must also remember the protective effect of CD40 expression on prostatic cancer cell lines (see Section 1.4.4.3). It is not difficult to imagine how such protection could lead to tumour progression.

However, there are also instances where the *lack* of CD40/CD40-L interactions seems to lead to the progression of cancer. CD40 is expressed in normal keratinocytes of the skin, but in basal cell carcinoma and in squamous cell carcinoma its expression is drastically down regulated (Viac *et al.*, 1997). In boys with the X-linked HIGM syndrome (see Section 1.4.4.1) there is a high incidence of carcinomas affecting the liver, pancreas and biliary tree. CD40 is normally expressed on these epithelial tissues but cannot be activated because of the congenital defect in CD40-L synthesis (Hayward *et al.*, 1997).

The clinical experience, therefore, parallels the conflicting evidence that is seen in *in vitro* experiments (see Section 1.4.4.3). The exact role of this surface molecule in epithelial cancers is still far from clear. Its function might vary from organ to organ and might depend on other ambient features e.g. the co-expression of other oncogenes. Further investigations are needed to clarify this situation.

1.5 A prelude to some experiments on lung cancer

As the preceding discussion shows, the role of apoptosis in cancer is a new and vibrant field of research. In lung cancer much work has been done on this subject but still many fundamental questions remain unanswered, for instance the correlation of apoptosis and the genes controlling it with survival, and with markers of disease severity. To attempt to answer some of these questions we undertook some investigations, taking advantage of a set of cases of lung cancer for which extensive information was available.

In 1991 D A R Boldy and his colleagues at the erstwhile East Birmingham Hospital (EBH) set out to investigate the role of interphase nucleolar organiser regions in squamous cell carcinoma of the bronchus in a 10 year follow up study of 138 cases. They identified patients treated by surgical resection of squamous cell carcinoma of the bronchus at EBH in 1977. After obtaining consent from the thoracic surgeons responsible for the care of the patients, they collected further information by examining the hospital case notes and the West Midlands Regional Cancer Registry forms. The data collected included the age and sex of the patient, the type of operation performed (lobectomy or pneumonectomy), histological diagnosis, pathological TNM staging and clinical outcome including cause of death. Boldy and his co-investigators then performed AgNOR staining on paraffin sections of the tumours. They also determined ploidy by DNA flow cytometry on suspensions of nuclei prepared from thick paraffin embedded sections. The results of this study were published in *Thorax*. (Boldy *et al.*, 1991)

We took this well defined and well documented series of cases and aimed to investigate some of the pathological parameters of apoptosis i.e. measurement of morphologically identifiable apoptotic bodies and the expression of apoptosis controlling genes.

We also wished to determine whether it was practical to assess apoptosis by PI staining in cultured tumour cells as this could potentially give data on the rate of apoptosis. We chose to begin our experiments with pleural effusions and then moved on to fresh resected lung tumours. The material for these experiments was obtained from patients admitted in three nearby hospitals - Birmingham Heartlands Hospital (formerly EBH), Walsgrave Hospital, Coventry and St. Cross Hospital, Rugby.

The outcome of these experiments will be discussed in the next few chapters.

CHAPTER 2

AIMS AND OBJECTIVES

2 Aims and Objectives

The aim of this project was to investigate the role of apoptosis and two apoptosis controlling genes (bcl2 and CD40) in squamous cell carcinoma of the lung.

1. We hoped to find a valid way of assessing apoptosis by morphological criteria in histological sections of lung cancer using Haematoxylin and Eosin (H&E) stain. The results obtained with this method were compared with that obtained by staining with monoclonal antibody to ASP (apoptosis specific protein).
2. We looked for correlation between the amount of observable apoptosis in histological sections and clinico-pathological parameters i.e. survival, disease stage, histological differentiation, proliferation rate (AgNOR) and DNA ploidy.
3. The expression of Bcl2 and CD40 in squamous cell lung carcinoma was quantified using immunohistochemical methods. We analysed if the expression of these proteins correlated in any way with clinical outcome (survival), with the amount of apoptosis in histological sections and with several established markers of disease severity e.g. disease stage, grade of differentiation, AgNOR and DNA ploidy. We also examined if the expression of one protein influenced the expression of the other.
4. We aimed to determine apoptotic rate in cultured tumour cells derived from malignant pleural effusions and from disaggregated freshly resected lung tumours. Our main means of quantifying apoptosis in these cells was calculation of sub-diploid fractions in DNA histograms obtained from flow cytometric analysis of cells stained by PI and BerEP4-FITC. To allow comparison, several other methods of quantifying apoptosis were also used in parallel i.e. analysis of H&E stained cytopins, analysis of histological sections stained with H&E and anti-ASP.

CHAPTER 3

INTRODUCTION TO EXPERIMENTAL METHODS

3 Introduction to experimental methods

Preface

To achieve our aims we conducted various experiments to identify and quantify apoptotic bodies and to identify and quantify the expression of two of the genes that control apoptosis. Our experimental material consisted mainly of formalin fixed, paraffin embedded histological sections of lung tumours. We also did experiments on malignant pleural effusions and freshly resected lung tumours—both of which are culturable.

The first section of this chapter is a critique of the techniques available for assessing apoptosis with special reference to those that we used in our experiments. Since we made extensive use of immunohistochemical methods throughout, the second section deals, in depth, with the principles underlying immunohistochemical staining.

3.1 The detection and measurement of apoptosis

Several different techniques are available to detect and quantify apoptosis—each based on some aspect of the biology of the process. As the forthcoming discussion will reveal each has its own limitations and therefore throughout our investigations we have used more than one method in parallel.

3.1.1 Morphological assessment with Haematoxylin and Eosin

Apoptosis had been originally defined as a morphologically distinct form of cell death that is characterised by cytoplasmic and nuclear condensation followed by fragmentation into small dense “apoptotic bodies” which are engulfed by neighbouring cells or

macrophages (see Section 1.2.2). Since the definition includes only morphological criteria it might be considered tautological to state that morphology is an invariant feature of this phenomenon. But it is all too easy to lose sight of this fact when faced with the huge amount of biochemical and genetic detail that has now emerged on the subject. It is also slowly emerging that some of this biochemistry and genetics is dispensable in individual cases and much of it is cell lineage specific or trigger specific. Therefore it might not be too far off the mark to claim that morphology is the *only* invariant feature of apoptosis.

Haematoxylin and eosin (H&E) is a widely employed staining technique that we used to detect and quantify apoptosis in paraffin embedded tissue sections and cytopsin preparations. The following criteria distinguish H&E stained apoptotic cells:-

1. Retracted often pink to orange cytoplasm;
2. Condensed nuclear chromatin:
 - a) earliest stage shows condensation along the nuclear membrane;
 - b) later uniformly condensed to round/oval, dark structures;
 - c) finally breaking up into several small, round, dark fragments;
3. Concerns isolated cells;
4. No inflammatory reaction.

In trying to quantify apoptotic cells with H&E there are several possible sources of error. Other cells that have condensed chromatin e.g. lymphocytes and mitotic bodies could be mistaken for apoptosis. Also small apoptotic fragments in the late stages are easy to miss. Both these mistakes can be substantially eliminated by counting at high magnification. In an elegant study on the methodology of counting apoptotic bodies in breast cancer, van de Schepop *et al.* have shown that counting at progressively higher magnification ($\times 400$, $\times 630$ and $\times 1000$) led to (a) higher apoptotic counts (more small apoptotic bodies were recognised) and (b) greater intra-observer and inter-observer reproducibility of results (less confusion with lymphocytes and mitoses) (van de Schepop *et al.*, 1996).

Apoptosis is a rare event in tissues (less than 1% in tumour sections) and there is a tendency for apoptotic bodies to cluster in adjacent fields. Thus if only a few fields are

examined, the count can vary widely from one examination to another. This variation can be eliminated considerably by increasing the size of the sample. As more and more cells are counted the variation decreases sharply and then plateaus off until counting more cells does not improve the results greatly. The “running mean” method can be used to determine the minimum number of cells to be counted for the results to be reliable. This method consists of calculating stepwise an apoptotic index (apoptotic cells /100 malignant cells) for every 100 malignant cells until the difference between the successive means becomes negligibly small (less than 1% of the index). In the lung cancer slides we found this to happen after counting 1500 cells. We found that examining 10 fields under the oil immersion lens was always enough to include 1500 cells. Again, van de Schepop *et al.* have shown that such systematic sampling protocols greatly enhance the reproducibility of results and is therefore mandatory for any quantitative analysis.

It is tedious to count such large numbers of cells, particularly in situations where apoptosis is a rare event, and several staining techniques have been devised to ease the process by enhancing the difference in staining intensity between condensed and normal chromatin. Examples of such special stains include:-

- (i) Feulgen's staining of Bouin's fixed tissue;
- (ii) Acridine orange stain;
- (iii) Gomori's silver methenamine stain;
- (iv) Differential staining with toluidine blue and safranin.

However these techniques cannot be applied to routinely fixed and processed specimens and have therefore not found wide usage. The annexin staining technique takes advantage of a biochemical change on the surface of apoptotic cells. Soon after the initiation of apoptosis phosphatidylserine (PS) is translocated from the inner face of the plasma membrane to the cell surface. Once on the cell surface PS can be easily detected by staining with an FITC conjugate of annexin V, a protein that has a strong natural affinity for PS (Martin *et al.*, 1995). It is impractical to use electron microscopy routinely although even the subtle changes in early apoptotic nuclei can be confidently identified by this method. Another approach is to immunostain specific protein markers or DNA strand

breaks characteristic of apoptosis. However such techniques have their own peculiar disadvantages as discussed below.

Compared with flow cytometry, counting in sections has the advantage of being able to select a specific morphological area for counting, thus avoiding benign cells.

Reviewing the various available methods of quantifying apoptosis a recent editorial in the *Journal of Clinical Pathology* (Harrison, 1996) favoured morphological assessment with H&E as the method of choice, provided the various caveats are kept in mind.

3.1.2 Detection of DNA strand breaks

Two special staining techniques have been developed that detect apoptosis by identifying the DNA strand breaks that are created by the activation of endonucleases (see Section 1.2.3.4 b). Both these methods can be used on formalin fixed, paraffin-embedded tissue sections. After protease treatment to permeate the tissue sections, biotinylated nucleotides are *in situ* incorporated into DNA breaks by DNA polymerase in the *in situ* end labelling or ISEL technique (Wijsman *et al.*, 1993) In the TUNEL [terminal deoxynucleotidyl transferase (TdT) mediated biotin-dUTP nick end labelling of DNA] technique (Gavrieli *et al.*, 1992) the enzyme TdT is used instead of polymerase. The labelled DNA is identified immunohistochemically with DAB via peroxidase-conjugated avidin (see Section 3.2.7). Intense staining is observed in nuclei or nuclear fragments with the morphological characteristics of apoptosis. This eases the recognition of scattered apoptotic cells and avoids confusion with other cells that have condensed chromatin e.g. mitotic cells or lymphocytes. These staining methods also allow the use of automated image cytometry for evaluating apoptosis. Furthermore, these techniques can be adapted for flow cytometric analysis using avidin-tagged fluorescein isothiocyanate (FITC) (see Section 3.1.5 e).

Although they facilitate the recognition of apoptosis, ISEL and TUNEL do not necessarily expand on the fraction of apoptotic cells that can potentially be identified by morphological criteria. Two studies have shown that ISEL and H&E staining recognise an almost identical proportion of cells as apoptotic (Ansari *et al.*, 1993; Wijsman *et al.*,

1993). This is because, contrary to previous opinion, the onset of morphological changes occurs simultaneously with or may even precede DNA fragmentation.

There are several situations in which non-apoptotic cells can stain positively by these methods. Normal cells have low levels of DNA strand breaks caused artifactually by fixation and processing, or even by the act of cutting the sections. But even these may be detected if pre-treatment with proteinase is slightly excessive. Since in necrotic cells the DNA is degraded by release of lysosomal DNAses, these cells incorporate the label as well. Because necrosis affects tracts of contiguous cells, in tissue sections it does not interfere with the identification of individual cells undergoing apoptosis. A variety of other biological processes may lead to positive ISEL staining, such as the nicking of DNA thought to occur during cellular differentiation. DNA damage caused by radiation or chemicals also leads to the incorporation of labelled nucleotides by DNA polymerase (Ansari *et al.*, 1993).

In other situations, cells which are morphologically obviously apoptotic may not take up any stain. It has been shown that in breast tissue, normal, tumourous or metastatic, ISEL fails to stain apoptotic cells whereas TUNEL stains them efficiently. It is proposed that these two staining methods work on different types of DNA strand breaks which in turn are produced by different types of endonucleases. Each tissue induces one specific kind of endonuclease and hence the difference in staining specificity (Mundle *et al.*, 1995). If apoptosis proceeds without internucleosomal fragmentation (as is now known to be possible - see Section 3.1.3 below) neither stain would work.

Thus it can be seen that these techniques are by no means specific or sensitive for apoptosis, and results must be interpreted with caution and correlated with morphological criteria of apoptosis.

3.1.3 DNA chromatin ladder

During the disintegration of the nucleus, initially large 50 kbp (kilo base pairs) and 300 kbp fragments are detectable transiently. In many cells there is further rapid and extensive double strand cleavage of internucleosomal DNA to yield a series of oligonucleosome

chains of 180-200 bp (base pairs) multiples (180 bp is the length of DNA in a single nucleosome). These are visualised as the characteristic “DNA ladder” on agarose gel electrophoresis. This is in contrast with the diffuse smear pattern of random DNA breakdown that occurs in necrosis.

It is now known that the nuclear changes of apoptosis can occur without oligonucleosome generation e.g. TGF β 1-induced rat hepatocyte apoptosis (Cohen *et al.*, 1992; Oberhammer *et al.*, 1992). Thus the “DNA ladder” is not as reliable a marker as it was once thought to be. Even when laddering *is* present, it does not help to quantify apoptosis.

3.1.4 Immunohistochemistry

We have made extensive use of immunohistochemical methods in our investigations. Immunology can aid in the analysis of apoptosis in two different ways.

3.1.4.1 Detecting the expression of genes controlling apoptosis

There are several genes which control apoptosis (see Section 1.2.3.5) of which we chose to investigate only two - bcl2 and CD40.

bcl2 is one of the key apoptosis-controlling genes. Its over-expression can block apoptosis in a wide variety cells. Bcl2 over-expression has been seen in many malignancies –both lymphoid and non-lymphoid –and is thought to have a significant role in the genesis of some cancers.

CD40 is a less well known player in the field of apoptosis. It is a cell surface receptor, structurally similar to the TNFR group of receptors. When it is bound by its ligand, CD40 prevents apoptosis in lymphocytes presumably with the help of Bcl2 or one of its family members. Though CD40 is known to be expressed in epithelial cells and carcinomas, its exact biological role in these cells has not been investigated completely yet.

3.1.4.2 Detecting apoptosis specific proteins in cells

The identification, by immunological means, of chemicals which are specifically expressed in apoptotic cells aids their ready detection and facilitates rapid and confident assessment of apoptosis. Several proteins have been used as immunological markers of apoptosis. Examples include clusterin (product of SGP-2 or TRPM-2), Cathepsin D and tissue transglutaminase. Most of these proteins happen to be cell lineage or apoptotic trigger specific. This imposes obvious limitations on their use, but if a particular protein is properly validated in the cell lineage being studied, it can prove extremely useful. In our investigations we used one such protein which has been found to be a reliable marker of apoptosis in some situations.

ASP This recently discovered cytosolic protein is expressed specifically within apoptotic cells. While investigating the role of c-Jun expression during apoptosis in Burkitt's Lymphoma (BL) cell lines it was observed that a protein of 45,000 molecular weight (M.W) could be detected at high level on Western blots with an antibody raised against a synthetic peptide equivalent to amino acids 73-87 of the human c-Jun protein [antibody—c-jun/AP-1(Ab-2)] (Grand *et al.*, 1995). This protein was not found to be expressed in viable cells or in cells dying by necrosis. It was named apoptosis specific protein (ASP).

ASP has been observed in apoptotic cells of diverse origin. In addition to BL cells, adenovirus early region 1-transformed human embryonic retinoblastoma cell line (Ad12 E1 HER10), baby rat kidney cell lines (Ad12 Acc1H HLBRK) and hamster fibroblasts have all been shown to express this protein. A variety of apoptotic triggers are capable of causing ASP expression e.g. serum starvation, ionomycin (a calcium ionophore), actinomycin D and cycloheximide (RNA and protein synthesis inhibitors), anti-immunoglobulin treatment and cold shock. Following UV or γ irradiation ASP can be detected only in the rodent lines, even though apoptosis occurs to the same extent in other cell types. Thus there is some degree of trigger specificity.

Irrespective of the trigger used an excellent correlation is observed between the onset of apoptosis and the upregulation of ASP. However, if Bcl2 is over-expressed within these cells, it not only prevents apoptosis but also blocks ASP expression. This would indicate

that ASP is upregulated at a point immediately prior to or coincidental with an irreversible step in the apoptotic process. This would tally with the observation that in cells, ASP upregulation is detected at very early stages and is maintained at extremely high levels as apoptosis progresses. In fact ASP (which is mainly located in the cytoplasm) is also found in late apoptotic cells including phagocytosed apoptotic cells which lack any DNA.

It is this predominant cytosolic location which first raised doubts about the true identity of this protein. C-Jun and c-Fos are immediate early gene products, transcription factors that are known to be involved in the activation of the apoptotic machinery. But their expression is confined to the nucleus and there are no reports to suggest gross over-expression of c-Jun at late times in apoptosis which is seen in the case of ASP. Antibodies raised against other parts of the c-Jun molecule [a.a. 209-225—Ab1; a.a. 46-59—JunB] show no reaction with ASP. Finally, ASP has a M.W. of 45,000 while that of c-Jun is 39,000. Thus, Grand *et. al.* have concluded that the partial structural similarity between ASP and c-Jun is entirely fortuitous. ASP is clearly distinct from c-Jun and unlikely to represent any related transcription factor.

The cytosolic location and the relative insolubility of the protein in non-denaturing buffers suggested that it may form part of the modified cytoskeleton on apoptotic cells. During apoptosis several structural proteins e.g. β -actin, tubulin and vimentin are up-regulated or down-regulated. ASP shares many similarities with β -actin. They solubilise under similar conditions and their molecular weights are similar (β -actin M.W—43,000). However, they do not cross react with each other's antibodies and have now been found to have quite distinct peptide structures. Thus ASP though closely associated with β -actin is clearly distinct from it.

It was thought that ASP might be a partially degraded product of nuclear lamins that is found in myeloid and epithelial cells undergoing apoptosis. This contention has also been proved wrong.

Whatever the identity of this protein, several features make it an ideal candidate for an apoptotic marker. It is expressed in apoptotic but not in viable or necrotic cells and it is identifiable from a very early to a very late stage of apoptosis. Panchalingam *et al.* have recently used the ASP antibody [c-jun/AP-1(Ab-2)] to detect apoptosis in formalin fixed,

paraffin embedded tissue sections of Burkitt's Lymphoma and reactive lymph nodes. In a comparative analysis they found that ASP antibody seemed to be more sensitive and detected higher numbers of apoptotic cells within sections than the ISEL or TUNEL techniques. Furthermore, apoptotic macrophages in the subcapsular region of reactive lymph nodes stained positive with anti-ASP but were not detected by the other two techniques indicating the greater inherent sensitivity of this antibody (Panchalingam *et al.*, 1996).

In our investigations we first established that anti-ASP was able to detect apoptotic cells of pulmonary origin. We then felt justified to use it to validate the apoptotic indices obtained from H&E staining (see Sections 4.2.4 and 5.2).

3.1.5 Flow cytometric analysis

The fluorescence activated cell sorter (FACS) is a versatile tool for the investigation of the attributes of individual cells within larger populations. For every cell, numerous light scattering and fluorescent properties, each reflecting an underlying structural, biochemical and genetic attribute of the cell, can be measured simultaneously. Later sub-populations of cells with distinct characteristics can be discriminated and analysed in isolation. All this is done with speed and statistical precision.

The method requires as its raw material a suspension of single cells or nuclei, which is usually obtained from fresh disaggregated biological tissues or cultured cells. In 1983, Hedley and co-workers introduced a method whereby thick (50µm) paraffin-embedded sections could be disaggregated with pepsin to produce a suspension of nuclei. This enabled archival samples to be examined flow cytometrically for DNA content and nuclear antigens (Hedley *et al.*, 1983). However, cell surface antigens cannot be examined.

A part of our investigation was to determine whether it was possible directly to analyse apoptosis in living tumour tissue. We planned to do this by disaggregating fresh lung tumours (see Section 4.2) and culturing the cells so that the kinetics of the process could be analysed. To do this it is necessary to be able to distinguish the tumour cells from the

non-epithelial stromal cells. We used the epithelial cell surface specific marker BerEP4 for this purpose.

Several structural and biochemical properties of apoptotic cells have been used to flow cytometrically discriminate them from viable and necrotic cells.

(a) Assessment of DNA stainability (content) by propidium iodide

Dual staining with propidium iodide (PI) and BerEP4-FITC was the main method that we used to assess apoptosis in cultured tumour cells obtained from malignant pleural effusions and disaggregated fresh lung tumours (see Sections 4.2.11, 5.5.1 and 5.5.2.1).

When a cell suspension is fixed with ethanol, stained with the DNA dye propidium iodide [or, for that matter, any other DNA fluorochrome e.g. ethidium bromide, acridine orange, 7-aminoactinomycinD (7-AAD), the Hoechst dyes (Hoechst 33258 and 33342) and DAPI] and flow cytometrically analysed for fluorescent DNA content, a distinct sub-population of cells with lower DNA fluorescence than the G0/G1 cells becomes apparent. This “sub-diploid” or “sub-G1” population has been shown to consist of apoptotic cells.

Why do these cells display less DNA than others? Activation of endonucleases in apoptotic cells cleaves DNA into single nucleosomes and oligonucleosomes which can diffuse outside the nucleus. Following fixation with ethanol when the cells are rinsed, these nucleosomes diffuse outside the cell resulting in a lowered DNA content as seen on the histogram. It has also been suggested that ethanol fixation, by causing a change in DNA conformation may reduce the accessibility of DNA to fluorochromes without altering total cellular DNA (Darzynkiewicz *et al.*, 1992; Telford *et al.*, 1994).

The sub-diploid fraction has been shown to consist almost entirely of apoptotic cells. Necrotic cells generally do not show an immediate reduction in DNA stainability and therefore remain confined to the G0/G1 compartment of the DNA histogram. Careful analysis of both propidium iodide fluorescence and forward scatter for several cell types has demonstrated that the sub-diploid region represents intact cells and is not debris.

The proportion of cells in this region correlates well with apoptosis measured by other means e.g. the presence of chromatin degradation as measured by gel electrophoresis, DNA separation assays and nuclear morphology as determined by electron micrography.

On gel electrophoresis the chromatin of flow cytometrically sorted sub-diploid cells appears to be almost entirely fragmented in roughly 200 bp multimers, compared with the sorted G0/G1 phase cells which have little or no fragmented DNA. Finally, the sub-diploid peak which is noticed when mouse thymocytes undergo apoptosis with glucocorticoids, disappears when the experiment is repeated with inhibitors of apoptosis (Tounekti *et al.*, 1995).

Thus, this method of staining provides a reliable assessment of apoptosis. The method is easy, rapid and convenient because fixed samples can be prepared and analysed at a later time. But perhaps the greatest advantage of fixed cell assays is the ability to combine fluorescent surface phenotyping with DNA staining permitting the detection of apoptosis in small sub-populations of cells within heterogeneous tissues.

Although ethanol is known to damage cell membranes, gentle ethanol fixation can preserve membrane structure and associated markers while still inducing the alterations in apoptotic chromatin responsible for reduced DNA dye fluorescence. In our experiments we labelled the heterogeneous tumour cells with a epithelial specific cell surface marker—BerEP4—prior to fixation and DNA staining with PI. This allowed us to analyse for apoptosis within the epithelial population ignoring the non-tumour cells.

BerEP4 is an antigen of unknown function which is found exclusively in epithelial cells (Latza *et al.*, 1990). The monoclonal antibody to BerEP4 is directed against the protein moiety of two 34 kilodalton and 39 kilodalton glycopeptide chains, which are not covalently bound. This antibody has been used to examine BerEP4 expression in a wide range of normal and malignant tissues.

All normal epithelial tissues express the antigen on the cell membrane and some in the cytoplasm as well. Only hepatocytes, gastric parietal cells and the superficial layers of squamous epithelia are negative. The BerEP4 antigen is not expressed in non-epithelial cells e.g. nerve, glial, muscle, mesenchymal or lymphoid tissue (see Section 5.5.2.3).

All carcinomas including squamous lung cancer stain positive with this antibody. In a small series examined by Latza *et al.* 3 out of 6 lung adenocarcinomas and 1 out of 5 oat cell carcinomas did not stain (Latza *et al.*, 1990). In another study on cells derived from malignant pleural effusions 57% of 23 pulmonary adenocarcinomas and 60% of 43

pulmonary carcinomas of all other histological types were BerEP4 positive (Illingworth *et al.*, 1994). The antibody does not stain non-epithelial tumours especially mesotheliomas. This has prompted several investigators to use it to differentiate between malignant mesotheliomas and secondary pleural adenocarcinomas—a difficult clinical and pathological problem (Dejmek and Hjerpe, 1994; Hartmann and Schultze, 1994; Sheibani *et al.*, 1991).

There are several other methods for assessing apoptosis some of which will be briefly mentioned here (for detailed review - Darzynkiewicz *et al.*, 1992).

(b) Plasma membrane integrity

In unfixed apoptotic cells (as well as in live cells) the plasma membrane is intact and can exclude dyes such as PI. This is in contrast to necrosis where one of the earliest changes is loss of membrane integrity so that these cells stain with PI. With the Hoechst dye 33342 live cells stain strongly, apoptotic cells less strongly and necrotic cells hardly at all.

The simultaneous use of these two dyes can help distinguish between apoptotic, necrotic and live cells.

(c) Cell organelles

Intact mitochondria in apoptotic cells can retain the dye Rhodamine 123 resulting in green fluorescence. In necrotic cells, mitochondrial swelling and disruption is one of the earliest changes and therefore Rhodamine 123 is not retained.

The ability of apoptotic cells to accumulate acridine orange in lysosomes (resulting in red fluorescence) is not markedly changed from live cells. Necrotic cells on the other hand tend to lose their ability to concentrate this dye in lysosomes resulting in minimal red luminescence.

(d) Light scatter

The light scattering properties of apoptotic cells are affected by the changes in the morphology of these cells namely chromatin condensation, nuclear fragmentation, cell

shrinkage and shedding of apoptotic bodies. Initially forward light scatter is reduced with minor increase in side scatter. Later both are reduced. In contrast, necrosis results in an immediate decrease of both forward and side scatter.

(e) DNA strand breaks

DNA strand break labelling using either DNA polymerase or TdT followed by FITC-conjugated antibodies can be used to detect apoptosis. However, as discussed above (Section 3.1.2), such strand breaks are not unique to apoptosis and the results need to be interpreted with caution.

(f) Protein content

In addition to lowered DNA, apoptotic cells contain less protein a property which can be detected flow cytometrically by staining with sulforhodamine 101.

(g) Sensitivity to DNA denaturation

The condensed chromatin of apoptotic cells is highly susceptible to denaturation, higher even than the DNA of mitotic cells. This property can be measured by careful staining with acridine orange.

Combinations of these various methods are often practised in an effort to increase the confidence in identifying apoptotic cells.

3.2 Immunohistochemical methods

3.2.1 Introduction

Immunohistochemistry can be considered as the demonstration of antigens in tissue sections by the use of specific antigen-antibody interactions which culminate in the

attachment of a marker to the antigen. Immunohistochemistry exploits the very specific binding exhibited by an antibody for its antigen. The reaction site can be identified in a specimen and associated with specimen structure by attaching a microscopically dense probe to the antigen-antibody complex. Proteins, carbohydrates, nucleic acids, lipids and many other naturally occurring and synthetic compounds can act as successful antigens for raising antibodies, and consequently there is a wide variety of molecules which can be correlated with specimen structure. Microscopical analysis by immunocytochemistry can therefore be used to obtain a unique view of macromolecular changes within individual cells. If the antibody is specific, immunocytochemistry can be performed on virtually any biological specimen provided (a) the antigen remains intact and able to bind the antibody and (b) the specimen preparation technique exposes the antigen for immunolabelling by the antibody.

Immunocytochemistry is highly specific, relatively quick and relatively sensitive. Many of the reagents are commercially available and the techniques are now routine. Immunocytochemistry is now being used in almost every area of biological research from routine diagnostic work through to complex research projects.

While the methodological details of the staining protocol are discussed in the methods section (see Section 4.2) what follows below is a discussion of the principles underlying the various steps. The several options available at each stage are discussed while mentioning the particular option that was chosen and the reasons for such a choice.

3.2.2 Tissue Fixation

A prerequisite for all histological and cytological investigations is to ensure preservation of tissue architecture and cell morphology by adequate and appropriate fixation. Furthermore, an ideal fixative should stabilise and protect tissues and cells from the damaging effects associated with subsequent treatment. Reagents used for this purpose may significantly diminish the antibody binding capability. The demonstration of many antigens depends heavily on the fixative employed and the immunocytochemical method

selected. Prompt fixation is essential to achieve consistent results. Poor fixation or delay in fixation causes loss of antigenicity or diffusion of antigens into the surrounding tissue. There is no one fixative that is ideal for the demonstration of all antigens and many antigens necessitate the use of frozen sections.

In general many antigens can be successfully demonstrated in formalin-fixed paraffin-embedded material. The most widely used fixatives in diagnostic hospital histology laboratories are formalin based; neutral buffered formalin, formal saline, or 10% formalin in tap water being the three most commonly employed for general use.

In cases where antigen demonstration is poor, improved immunocytochemical staining may be achieved by the use of specially formulated fixatives. Examples include the mercuric chloride based fixative B5 which is recommended for the fixation of lymph node biopsies. Periodate-lysine-paraformaldehyde (PLP) is a fixative designed to protect the carbohydrate moiety associated with cell membranes against the damaging effects of tissue processing and embedding. PLP is said to give a better demonstration of membrane associated antigens than formalin fixation.

All the specimens that we used had already been fixed in formalin and embedded in paraffin.

3.2.3 Pre-Treatment

Tissue fixation in formalin results in cross linking of proteins via aldehyde groups reacting with cellular nucleophiles (for example, NH₂ groups on amino acids or nucleic acids) to produce methylene bridges. To reverse this cross linking and hence make the tissue amenable to molecular and immunohistochemical analysis, the sections are usually pre-treated with a proteolytic enzyme. Several such enzymes are in use e.g. proteinase K, trypsin, chymotrypsin and pepsin. The main problem encountered with this proteolytic procedure is that because of differences in tissues and in tissue fixation times, cross linking of proteins can occur to differing degrees. As a result, enzyme concentration, digestion time and temperature, which are all critical parameters, normally have to be optimised for each tissue preparation. Furthermore, tissue digestion may sometimes destroy various

surface molecules on cells, such as cluster of differentiation (CD) antigens, which may then interfere with their detection by immunostaining. A high concentration of proteolytic enzymes can also lead to false positive or negative detection of apoptosis, as well as to high non-specific background staining and destruction of tissue morphology.

To overcome these problems several non-enzymatic alternatives have been used successfully. One of these involves pressure cooking the tissue sections prior to staining. We used the other alternative i.e. microwaving the tissue sections for 15 minutes in citrate buffer. We found that this procedure was simple, less time consuming and resulted in more consistent and enhanced staining, while not requiring complicated, expensive equipment.

3.2.4 Quenching endogenous peroxidase

A crucial step of immunostaining involves the conversion of a colourless chromogen to a coloured product by the enzyme peroxidase (see Section 3.2.8 below). To ensure that the staining produced is specific, peroxidase is added linked to an immunoreagent. However, peroxidase is a normal component of many tissues like macrophages and red blood cells and may lead to non-specific staining if its activity is not destroyed. Endogenous peroxidase activity can be inhibited with excess of its substrate, hydrogen peroxide, before beginning the immunoreaction.

There are several ways of doing this. Sodium azide and nascent hydrogen peroxide is a mild blocking agent while a stronger method involves the use of periodic acid. We used the commonest method which involves incubating the tissue sections in 0.3% H_2O_2 in methanol.

3.2.5 Prevention of non-specific staining

Non-immunological attachment of antibodies and dense markers to the specimen can result in non-specific staining. This can be reduced by reagents added to the buffers. These reagents are of three types:

- (a) Surfactant reagents such as Tween 20 and NaCl, reduce surface charges on the specimen, thereby reducing electrostatic attachment of molecules to the specimen.
- (b) Chemical reagents, such as 0.02 M glycine, or 1% sodium borohydride block free aldehyde groups on the tissue thereby preventing fixation of proteins to the specimen.
- (c) Blocking reagents such as bovine serum albumin are proteins which compete with the immunological reagents for non-immunological sticky sites on the specimen.

We used an appropriate blocking serum for all our staining procedures. The tissue sections were incubated in blocking serum just prior to the addition of the primary antibody.

3.2.6 The antibody

The antibody is central to the success of immunocytochemical staining. Antibodies can be raised against any biological macromolecule such as proteins, carbohydrates, lipids and nucleic acids. A good antibody is highly specific, possessing a high titre (amount of antibody activity), high affinity (the exactness of stereochemical fit of an antibody-combining site to its complementary antigenic determinant) and high avidity (the total binding strength of a multivalent antigen with the antibody). Both polyclonal and monoclonal antibodies can be used for immunocytochemistry (Beesley, 1993).

Polyclonal sera contain a mixture of high affinity antibodies, each active against different epitopes on the antigen. Production of polyclonal antisera is relatively simple and of low cost. An antigen is injected into an host animal, usually either rabbit, sheep or goat and, after booster injections, the serum is periodically collected and tested for antibody. Antibodies, which are glycoproteins are produced in the spleen of the host animal by B lymphocytes and plasma cells, the latter being the terminal stage in the differentiation of B lymphocytes. The antiserum will probably contain antibodies to impurities in the immunogen. Antibodies raised against the contaminating immunogens are often of low titre and affinity and can be diluted out to zero activity for immunolabelling. In consequence of the high possibility of a wide spectrum of antibodies being present in the host animal in response to previous antigen challenges, serum removed from the animal before injection of the immunogen is important as a negative, or pre-immune, control.

A monoclonal serum is a 'pure' preparation of one of the constituents of polyclonal antiserum. Monoclonal antibodies are specific to a single epitope on the antigen. Monoclonal antisera are useful as diagnostic reagents and for immunocytochemical research since large amounts of antibody with narrow specificity, titre and homogeneity in all batches can be produced. A low antigen dose is needed for immunoresponse and the antigen need not be purified with as much stringency as for the production of polyclonal antisera. The initial phase of preparation is to induce synthesis of a polyclonal antiserum in the host animal as described above. The plasma cells are removed from the host animal and each fused with a malignant myeloma cell to form hybridoma cells. Hybridoma cells are isolated and cultured and theoretically each will produce unlimited monoclonal antibodies raised against a specific epitope, each antibody being identical in reactivity and titre. These hybridoma cells are either cultured *in vitro*, in which the tissue culture supernatant fluid contains antibody, or *in vivo* in the peritoneal cavity of a suitable host and it is the ascites fluid which contains antibody.

Whatever type of antibody is used it is important to be absolutely certain of its class and sub-class since this will determine the other reagents to be used.

3.2.7 Immunolabelling methods

After the primary antibody is attached to the antigen in question the next step is to label the antibody with a microscopically dense marker in order to make it visible. There are several ways of doing this (Jackson and Blythe, 1993).

(a) Avidin-biotin methods

Because of its reliability and ease of performance this is the method that we used in our experiments. The technique utilises the high affinity that is displayed between avidin, a basic glycoprotein (M.W. 68 kDa) and the small (M.W 244 Da) water soluble vitamin biotin. For technical reasons avidin is often substituted by streptavidin.

A secondary biotinylated antibody raised against the primary antibody is added to the sections. Next, a complex formed between streptavidin and horseradish peroxidase is

added, which, because of its high affinity for biotin attaches itself very strongly to the secondary antibody. This results in the formation of a peroxidase-streptavidin-biotin complex, the so called ABC complex, at the antigen binding site. Since many biotin molecules can be attached to a single molecule of secondary antibody there is a several fold amplification of the signal at the antigen site.

(b) Enzyme-anti-enzyme methods

These indirect methods utilise a pre-formed, cyclic, enzyme-anti-enzyme immune complex composed of three enzyme molecules and two antibody molecules. The methods are named after the particular enzyme-antibody complex that is used in the technique. The commonest used are the peroxidase-anti-peroxidase (PAP) technique and the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique. These methods are very sensitive and can be used for both paraffin processed material as well as for frozen sections.

(c) Immunogold methods

Immunogold reagents consist of immunological reagents adsorbed on to the surface of colloidal gold particles. Considerable amplification of the signal can be obtained by silver enhancement of the colloidal gold particles and this is often used for specimens to be examined light microscopically.

(d) Protein A method

Protein A derived from the cell wall of the bacterium *Staphylococcus aureus* binds to the Fc portion of IgG molecules from several mammalian species. Protein A may be used as a secondary reagent for the demonstration of antigens by light microscopy when labelled with suitable enzymes or colloidal gold.

(e) Direct method

In this technique a labelled antibody reacts directly with the antigen in the histological or cytological preparation. This method utilises only one antibody and can be quickly completed. The technique however provides little signal amplification and is rarely used on paraffin sections. The main application remains in immunofluorescence, where this

technique is used to identify immunoglobulin and complement in frozen sections of skin and renal biopsies.

(f) Two step indirect method

An unlabelled primary (first layer) antibody is visualised by a labelled secondary (second layer) antibody directed against the immunoglobulin of the animal species in which the primary antibody had been raised. This method is more sensitive than the direct method because several secondary antibodies may react with different antigenic sites on the primary antibody.

3.2.8 Chromogens

Except for the colloidal gold methods, immunocytochemical staining methods for light microscopy depend upon enzyme-substrate reactions which convert colourless chromogens into visible, coloured end products. Since the introduction of horseradish peroxidase as an antibody label, reliance has been placed on the sensitive hydrogen peroxide-diaminobenzidine reaction, which produces a brown end product insoluble in alcohol, xylene, and other inorganic solvents. When freshly prepared diaminobenzidine (DAB) solution is added to the tissue section the peroxidase at the antigen binding sites converts it to a brown end product. Thus the antigen site is distinctly labelled.

3.2.9 Validation

It is possible to achieve 'credible' non-specific labelling on a specimen. Likewise the absence of immunolabelling does not always signify the absence of antigen. Controls are therefore absolutely essential and should be devised specifically to verify each experiment. A positive control is a tissue specimen that is known to contain the antigen in question. The same tissue can be used as negative control if the primary antibody is omitted from the staining protocol.

3.2.10 Quantitation

It has been widely reported that tissues display heterogeneity after immunostaining. Of the tumours that we stained, only a small fraction were uniformly positive or negative. The majority were heterogeneous with varying proportions of stained and unstained cells and also varying strengths of staining (see Sections 5.4.1 and 5.4.2).

It is possible that this could be an artefact. During immunostaining, unless one is careful, air bubbles can form on the surface of the slide and prevent the reagents from coming into contact with the tissue. This can result in patchy areas of (false) negative staining. That this was definitely not the case is proved by the fact that even within negatively stained tumour cells, infiltrating lymphocytes and macrophages often stained strongly positive proving that the dyes had actually reached the area in question.

Therefore, one must interpret such differences in staining within a tissue to represent a real difference in protein expression among cells. It is likely that in the late stages tumours consist of a heterogeneous population of cells each group with slightly different phenotypic and genotypic characteristics and each the progeny of a different ancestor that arose with successive mutations during tumour progression.

Because of numerous confounding staining variables it is generally considered prudent not to equate intensity of staining with strength of gene expression. Rather the proportion of positively staining cells might be a better guide. Some investigators use a semi-quantitative overall general impression to define whether a tumour is positive or negative for a given antigen. The majority prefer to count the numbers of stained and unstained tumour cells within a section to calculate an index of percentage positive cells. Again there is no consensus about how to interpret such an index and different investigators have arbitrarily used different cut-off points. Values of 30%, 20% and even as low as 2% or 1% have been variously used to define positivity.

In our series we analysed the distribution of positivity for immunostaining among the tumours and the cut-off point was determined based on this distribution (see Sections 5.4.1 and 5.4.2.)

CHAPTER 4

MATERIALS AND METHODS

4.1 Materials

The main material used for this investigation was two series of lung tumours: one archival, for which extensive information was available but for which fresh tissue was not; and the other current, as a source of fresh tissue which could be cultured but for which much other data e.g. length of post operative survival was not available. Paraffin blocks were available for the current series of lung tumours. In addition some malignant pleural effusions were also used. This material was in the form of fluid drained from the chest cavity.

4.1.1 Paraffin blocks

(a) The archival material used for our investigations was 134 paraffin blocks containing squamous lung tumours that had been resected in 1977. These cases were reported by Boldy *et al.* in 1991 as discussed in Section 1.4.7. We could find all but four of the original 138 paraffin blocks from the archives of the pathology department of Birmingham Heartlands Hospital (BHH). From each of these 134 blocks, three sections of 5 μ m each were prepared on Vectabond (Vector Laboratories Inc.) coated Surgipath slides for staining with Haematoxylin and Eosin, and antibodies to Bcl2 and CD40. A random block of 26 cases was chosen from which an additional 5 μ m section was obtained for staining with anti-ASP antibody. The clinical and pathological details of these patients are tabulated in Appendix C.

(b) We also obtained paraffin blocks of the 21 patients whose fresh lung tumours we used for cell culture experiments (see below - Section 4.1.3). From each of these blocks four 5 μ m sections were obtained on Surgipath slides coated with Vectabond for staining with Haematoxylin and Eosin, antibodies to Bcl2, CD40 and ASP. Details of these patients are given in Table 5.33 in Section 5.5.2.

4.1.2 Malignant pleural effusions

Pleural fluid was collected for experiments from 10 patients after consent had been obtained both from the patient as well as the physician concerned. Four of these patients were from Walsgrave General Hospital (WGH), Coventry and six were from St. Cross Hospital, Rugby. Fluid was taken only from those patients whose effusions had proved to be malignant by fluid cytology or pleural biopsy or both. None of the patients had had any chemotherapy prior to fluid collection. Details of these patients have been listed in Table 5.32 in Section 5.5.1. Not all of these patients had primary bronchogenic carcinoma. Some had secondary involvement of the pleura from a primary elsewhere. However, this did not detract from the initial purpose of our experiments.

Between 1-1.5 litres of pleural fluid was aspirated from each patient. Immediately after aspiration the fluid was transferred into sterile containers and transported to the Biological Sciences laboratory at Warwick University. It usually took no more than 20 minutes to drive from either hospital to the University.

4.1.3 Fresh resected lung tumours

For our experiments 21 fresh resected lung tumours were collected from BHH and one from WGH, Coventry. Many of the patients operated at BHH had been referred from other peripheral hospitals. In some of these cases histology could not be determined pre-operatively in spite of all effort (bronchial lavage cytology, percutaneous X-ray guided biopsy). In these cases lung resection was undertaken on the basis of strong clinical and radiological suspicion of malignancy.

The full histology report was obtained later to determine the tumour type, differentiation and pathological stage. These details are tabulated in Table 5.33. One of the cases (LC16) turned out not to be a tumour at all but a fungal infection - actinomycosis. The rest of the group comprised diverse pathological types of lung cancer but the majority (55%) was squamous cell carcinoma.

Once the lung, or a lobe, was resected, the specimen was immediately taken to the histopathologist who examined the sample and chose a representative area avoiding necrosis and normal lung tissue. Then, sugar cube sized portions of tumour (each about 1 cm³) were cut out for our experiments. These cubes of tissue - usually 3 or 4 per tumour, occasionally 2 - were immediately transferred into 20ml universals containing cold RPMI medium to preserve viability during transport. The universals were placed in a Dewar's flask containing ice and immediately transported to the Biological Sciences laboratory at Warwick University. This journey took between 30-45 minutes depending on traffic on the A45.

I am very grateful to Dr. S Jane Darnton, senior research scientist at the Oesophageal Research Laboratory, BHH, for her tireless help with identifying and collecting the tumour samples.

To corroborate and expand on the results obtained from the fresh lung tumours, the corresponding paraffin blocks were obtained and examined as described in Section 4.1.1(b).

4.2 Methods

Introduction and Scheme of experiments

The various experimental methods that we used are described in this section as a series of protocols.

As mentioned before paraffin sections were stained with H&E, antibodies to Bcl2, CD40 and ASP. The staining methods are described in protocols 1-4.

Pleural effusions and fresh lung tumours were processed to obtain a single cell suspension that could be cultured. The aim was to carry out serial measurements of apoptosis on these cultured tumour cells by (a) counting morphologically apoptotic cells on H&E stained cytopins and (b) measuring the sub-diploid fractions on DNA histograms obtained by FACS analysis. These methods are described in protocols 5-13.

Before that a flow chart is given (overleaf) describing the sequence of the experiments conducted on lung tumours. For pleural effusions, with one exception, an identical scheme was followed. There was no need to disaggregate effusions as the cells were already in suspension. In fact this was the main reason why we started our experiments with effusions rather than solid tumours.

Key to the flow chart

After disaggregation and density centrifugation to remove RBC, a mixture of tumour cells, stromal cells and lymphocytes was obtained.

At 0 hours the following experiments were done with this cell suspension.

(a) Cytopins were obtained for staining with H&E and BerEP4.

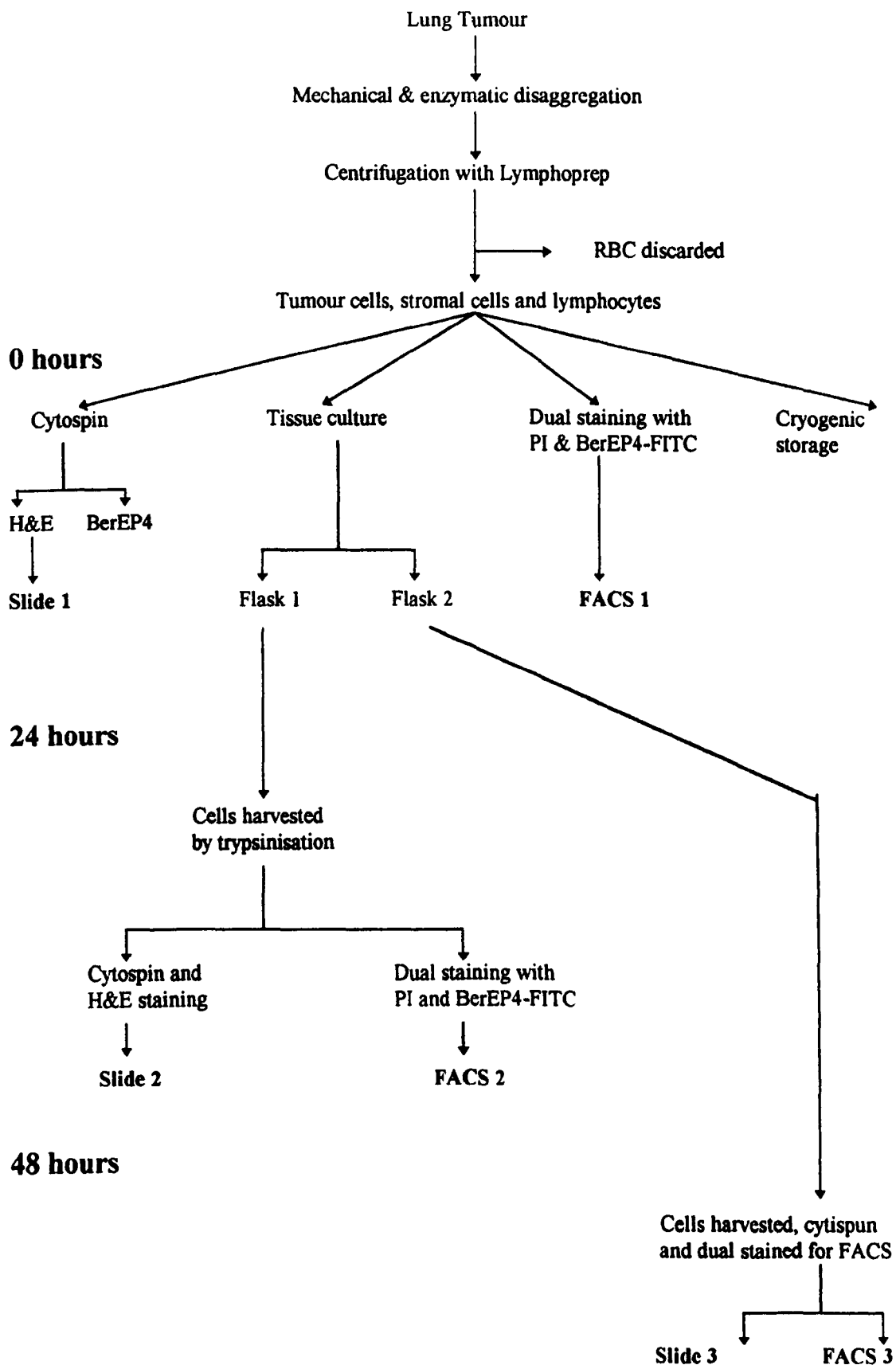
(b) Cell culture was started in two flasks containing complete RPMI medium.

- (c) An aliquot of cells was fixed and stored at 4°C to be dual stained with PI and BerEP4-FITC for FACS analysis later.
- (d) A portion was set aside for freezing in liquid nitrogen.

At 24 hours cells were harvested from one of the flasks. Cytospins were obtained for H&E staining. A portion of the cells was dual stained with PI and BerEP4-FITC.

At 48 hours cells were harvested from the other culture flask and processed as at 24 hours.

Thus, at the end of the experiments, three H&E stained slides and three flow cytometric samples were available for parallel measurements of apoptosis. The corresponding paraffin embedded histological sections provided information about the expression of Bcl2 and CD40 and apoptosis measured by H&E and ASP. These results are discussed in Section 5.5.



4.2.1 Haematoxylin and Eosin staining of tissue sections

1. Paraffin sections were dewaxed by soaking them twice in a xylene bath for 5 minutes each time.
2. Xylene was removed by treating with 90% alcohol and then with 70% alcohol each for 3 minutes.
3. Sections were then transferred to water
4. Stained with Harris' haematoxylin (Sigma) for 10 minutes.
5. Washed in water for 3 minutes.
6. Dipped in acid alcohol (1% hydrochloric acid and 70% alcohol) for 10 seconds.
7. Washed in water for 2 minutes.
8. Sections "blued" by dipping in 2% sodium bicarbonate for 30 seconds.
9. Washed in water for 3 minutes.
10. Stained in 1% Eosin (Sigma) for 5 minutes.
11. Washed briefly in water for 30 seconds.
12. Dehydrated by passing through ascending grades of ethyl alcohol (70%, 90%) to absolute ethyl alcohol—for 2 minutes in each.
13. Alcohol removed by clearing the sections in xylene for 3 minutes.
14. Finally, sections mounted with cover-slips using DPX (Sigma).

4.2.2 Staining tissue sections with Bcl2

1. Dewaxing and hydration of sections was carried out as described in Steps 1-3 of Protocol 1.
2. The sections were then placed in a microwave-proof plastic bowl and immersed in 10% citrate buffer (pH = 6.0). The bowl was covered with cling film which was punctured to allow steam to escape. This was boiled in a microwave at full power (750W) for 15 minutes and allowed to cool for 10 minutes.

3. Washed in PBS for 5 minutes. Endogenous peroxidase activity quenched by soaking in 0.6% H₂O₂ in methanol for 15 minutes.
4. Washed in water for 5 minutes.
5. Slides were next transferred to a Sequenza (Shandon Scientific) and washed with PBS for 5 minutes.
6. Three drops of diluted normal blocking serum (Vectastain Elite kit, diluted in PBS) were added and incubated for 10 minutes.
7. 150µl of primary anti-Bcl2 antibody was added to the slides and incubated for 30 minutes.

Antibody specifications:-

Monoclonal mouse anti-human bcl2 antibody

Clone 124

Isotype IgG1, kappa

Dilution 1:150

Source Dako (High Wycombe)

8. After a 5 minute PBS wash, 3 drops secondary biotinylated antibody (Vectastain Elite kit, diluted in PBS) were added and incubated for 30 minutes.
9. After another wash the slides were incubated for 30 minutes with 3 drops ABC reagent (Vectastain Elite kit, diluted in PBS)
10. The slides were once more washed in PBS before incubation for 1-3 minutes in the peroxidase substrate solution (DAB Substrate Kit for Peroxidase, Vector Laboratories Inc.).
11. Slides rinsed in cold running water and then counter-stained for 20-30 seconds in Mayer's haematoxylin (Sigma).
12. Slides allowed to stand for 5-10 minutes in cold running water to "blue up" and wash off excess haematoxylin.
13. Slides passed through 70% ethanol, 100% ethanol and xylene (2 minutes in each) before mounting in DPX (Sigma).

4.2.3 Staining tissue sections with CD40

1. Staining was carried out exactly as described in Protocol 2 for Bcl2 except that step 2 was omitted. There was no need for microwave pre-treatment for the antibody that we used.

2. Antibody specifications:-

Mouse ascites antibody G 28.5

Dilution 1:1000

Source Prof. L. Young

Cancer Research Campaign, Birmingham

4.2.4 Staining tissue sections with ASP

1. Staining carried out exactly as described in Protocol 2 for Bcl2.

2. Antibody specifications:-

Polyclonal rabbit antibody against c-jun [c-jun/AP-1 (Ab-2)].

Lot No. 3095-03-03

Dilution 1:50

Source Oncogene Science (Cambridge).

4.2.5 Disaggregation of tumours

1. Tumour tissue was placed in a standard 8.5 cm petridish (Sarstedt Ltd.) containing 2mls of RPMI medium (Gibco BRL).
2. Adherent pleura and small bronchial airways carefully dissected out. Specimen finely minced using a pair of forceps and a scalpel pre-sterilised in absolute alcohol.

3. Contents of petridish transferred into a 50ml universal (Sarstedt Ltd.) containing 10 mls of complete RPMI to which the following enzymes had been added:-

Collagenase (Boehringer-Mannheim)	2 mg/ml;
Hyaluronidase (Boehringer-Mannheim)	0.04mg/ml;
DNAse (Boehringer-Mannheim)	0.04 mg/ml.

4. Enzymatic digestion at room temperature for 2 hours with continuous gentle mechanical agitation in a tissue rotator.
5. Digested tissue filtered through a 70µm Falcon cell filter (Sarstedt Ltd.). A small aliquot of the filtrate examined microscopically in a haemocytometer to ensure formation of a single cell suspension. If necessary digestion allowed to continue for another 30 minutes.
6. Filtrate centrifuged at 1200 rpm, brake set3 for 5 minutes in Mistral 2000 MSE.
7. Supernatant discarded and pellet resuspended in complete RPMI (Gibco BRL).

4.2.6 Density centrifugation

- 1 10 mls of Lymphoprep (Nycomed) poured into an universal container (Sarstedt Ltd.).
- 2 10 mls of the tumour digest obtained at the end of Protocol 5 gently layered on top of Lymphoprep.
- 3 Centrifuged at 2000 rpm, brake set zero, for 25 minutes in a Mistral 2000 MSE.
- 4 Buffy coat at the interface of the bi-layer gently pipetted out and resuspended in RPMI medium. RBC at the bottom of the universal discarded.
- 5 A small aliquot was examined using a Neubauer haemocytometer and a cell count obtained. For each of the subsequent steps the cell suspension was diluted down appropriately.

4.2.7 Cytospin preparation

- 1 About 5,000 cells in 100µl of RPMI medium placed in each cytospin chamber.

- 2 A Shandon Cytospin 3 (Shandon Scientific) was used at 600 rpm for 3 minutes to spin cells on to Vectabond coated Surgipath slides.
- 3 Slides air-dried and fixed by dipping in 50% methanol and acetone for 5 minutes

4.2.8 Staining cytopins with Haematoxylin and Eosin

1. Slides with fixed cytospin preparations were washed in water.
2. Stained with Haematoxylin and Eosin as described in steps 4-14 of protocol 1.

4.2.9 Staining cytopins with BerEP4

1. Slides with fixed cytospin preparations were washed in water.
2. Staining was carried out exactly as described in Protocol 2 for Bcl2 except that step 2 was omitted. There was no need for microwave pre-treatment for the antibody that we used.
3. Antibody specifications:-

Purified monoclonal mouse antibody to BerEP4

Clone	BerEP4
Isotype	IgG1, kappa
Dilution	1:50
Source	Dako (High Wycombe)

4.2.10 Tissue culture

1. All operations were carried out using standard sterile techniques in a laminar flow cabinet (Gelaire BSB 4A, Flow Laboratories). The cabinets were routinely sterilised overnight by exposure to ultra-violet radiation and then washed with disinfectant (10% Savlon) and 70% ethanol prior to use. Periodic fumigation with formaldehyde was also carried out.

2. From the cell suspension obtained at the end of protocol 5 two aliquots of about a million cells each were added to two separate culture flasks. Cell culture was carried out in bicarbonate buffered Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL) which was supplemented with 10% (v/v) foetal calf serum (FCS), L-Glutamine (2mM) penicillin (500IU/ml) and streptomycin (500µg/ml). All cells were grown in tissue culture grade flasks of 75 cm² or 150cm² (Nunc.). These contained 25ml or 50ml of medium respectively. The flasks were incubated at 37°C in a humidified incubator containing 5% CO₂ in air.
3. At 24hrs and 48hrs respectively, cells from each of the flasks were harvested for analysis. Some of the cells were in suspension in the medium while a variable proportion were adherent to the culture flask. To collect the cells in suspension the medium was aspirated and centrifuged at 1200rpm for 5 minutes.
4. The adherent cells were removed by trypsinisation. Following aspiration of the medium culture flasks were rinsed briefly in 25mls PBS. Ten millilitres of Trypsin-EDTA solution [0.5mg/ml trypsin, 0.2mg/ml ethylene-diamine-tetra-acetic acid (EDTA), Gibco-BRL] was then added and the flasks incubated at 37°C until the cells had detached (1-5 minutes). The cell suspension was mixed gently with 10ml complete medium and the cells recovered by centrifugation at 1200rpm for 5 minutes.
5. The pellet was resuspended in fresh medium and added to the pellet obtained previously from the cells in suspension. This mixture of cells was then used for all further tests e.g. cyto-spin staining and flow cytometric analysis.

4.2.11 Dual staining with PI and BerEP4-FITC

1. About 500,000 cells aliquoted into an Eppendorf tube.
2. This was centrifuged at 1000rpm for 2 minutes (MSE Mistral 6L centrifuge) and the supernatant discarded. The rest of the procedure was carried out with minimum exposure to light to prevent quenching of the fluorescence label.
3. Pellet of unfixed cells suspended in 20µl of BerEP4-FITC and incubated at 4°C for 20 minutes.

Antibody Specifications:-

FITC-Conjugated monoclonal mouse Anti-human epithelial antigen

Clone BerEP4

Isotype IgG1, kappa

Source Dako (High Wycombe)

4. After 20 minutes unbound antibody was discarded by washing with PBS, centrifuging at 1000rpm for 2 minutes and aspirating the supernatant.
5. Cells fixed by adding 1ml of ice cold 70% ethanol and incubating at 4°C for 10 minutes.
6. Excess ethanol washed off with PBS as above.
7. Pellet resuspended in 1ml of Propidium Iodide solution [Propidium Iodide (Sigma) 100µg/ml, RNase (Boehringer-Mannheim) 20µg/ml].

4.2.12 Cryogenic storage and retrieval of cells

1. Cells pelleted and resuspended in RPMI containing 10%(v/v) dimethyl sulfoxide (DMSO, BDH) at an approximate concentration of 5×10^6 cells/ml.
2. Mixture transferred to a freezing vial (Costar Ltd.), wrapped in paper tissue and frozen at -70°C overnight.
3. The next morning the vials were transferred to liquid nitrogen.
4. To recover frozen cultures, vials were thawed in a 37°C water bath for 1-2 minutes. The contents were transferred to a universal containing 5mls of complete RPMI medium (warmed) and pelleted to remove DMSO.
5. The cell pellet was resuspended in 5ml complete medium and transferred to a 25cm² tissue culture flask.

4.2.13 Flow cytometric analysis

Flow cytometric analysis of these cells was carried out using a Beckton Dickinson FACStar. This was operated as described in the users manual. To standardise the settings before each operation, stained and unstained (fixed) cells from two cell lines were used—HL60 a myelogenous cell line and OE33 an oesophageal cancer derived cell line. HL60 served as a negative control for BerEP4 staining while OE33 was the positive control. Both cell lines were maintained in culture continuously to serve as standards for FACS analysis.

Typically 5,000 events were collected from each tumour sample. All data were collected in list mode and four parameters were checked; forward scatter-FSC, side scatter-SSC and fluorescence 2-FL2 (i.e. PI staining) were collected in linear amplification. Fluorescence 1-FL1 (i.e. BerEP4 staining) data were collected in logarithmic amplification.

Typical settings used were:-

<u>Parameter</u>	<u>Voltage</u>	<u>Amplification</u>
FSC	0	16
SSC	300	8
FL1	600	log
FL2	500	8

4.2.14 Statistical analysis

All statistical analysis was done with the help of C-Stat, a software programme published by Cherwell Scientific Publishing Limited, Oxford. For the comparison of means between two sets of data the Mann Whitney U test was used because the data were not normally distributed. For tests of association, regression analysis was done, or Spearman Rank test if the data were not normally distributed.

CHAPTER 5

RESULTS

5.1 Assessment of apoptosis by morphological criteria in 134 cases of squamous cell carcinoma of the lung

Introduction

As discussed in Section 1.2.5 apoptosis takes place in tumours and it is important to try and quantify it. There are several ways of identifying and assessing apoptosis in histological sections. One could use immunohistochemical methods to identify DNA strand breaks or apoptosis specific proteins in cells. We chose the much simpler method of counting apoptotic bodies in H&E stained sections as the main method for establishing apoptotic index. As discussed in Section 3.1.1 this method is easy, cheap, widely available and can provide a reliable index. To quantify apoptosis we have taken advantage of a set of cases of lung cancer for which there was already extensive information about pathological parameters and clinical outcome (see Section 1.4.7 and Appendix C).

Results

Paraffin sections were stained with haematoxylin and eosin as detailed in Section 4.2.1. Under the microscope the sections showed areas of neoplastic tissue of squamous differentiation surrounded by fibrous tissue stroma (Fig. 1.1). Normal lung tissue was often seen at the edge of the section. The malignant cells were easily distinguishable from normal alveolar cells by their larger size and hyperchromatic nuclei. In the neoplastic areas normal lung architecture was replaced by tissue of squamous differentiation with stratification, formation of intercellular bridges and intracellular keratinization.

Apoptotic cells were counted only in the neoplastic areas taking care to avoid normal epithelium and fibrous stroma. Also areas of necrosis were avoided. If any necrotic cells, stromal cells or normal epithelial cells abutted in the field being examined these cells were not counted.

Apoptotic cells are identified by the following criteria:- a) cells showing marked condensation of chromatin and cytoplasm b) cytoplasmic fragments containing condensed chromatin c) intra- and extracellular chromatin fragments (Fig. 1.2).

Counting was always done under oil immersion producing a magnification of $\times 1000$. Before formal counting was undertaken the minimum number of cells to be counted was determined on a sample slide by the standard running means method (see Section 3.1.1). Using this method we determined that counting 1500 cells or 10 oil immersion fields would ensure that there was minimum intra-observer variation.

Thus 10 fields were chosen at random (observing the exclusion criteria explained above) from different parts of the tumour section. The number of apoptotic cells and the number of malignant cells in each field were recorded. Apoptotic bodies occurring in distinct groups and likely to be originating from one and the same cell were recorded as one apoptotic cell. The count in all 10 fields were added and expressed as an apoptotic index i.e. apoptotic cells/10,000 malignant cells. Although it would have been much easier, we decided to avoid the more popular method of quantifying apoptosis i.e. per mm^2 or per number of microscopic fields. Cancer cells vary widely in size from tumour to tumour and also within the same tumour and therefore the number of malignant cells per unit of area is likely to be highly variable. Accordingly, the score of apoptosis per area would tell us little of the frequency of apoptosis per number of cells, which is of much greater biological significance.

The value of the apoptotic indices obtained for all 134 cases ranged from 2.4/10,000 malignant cells to 145.5/10,000 malignant cells with a mean of 30.29, S.D of 24.75 and median 22.3.

To check whether the results were indeed reproducible, each slide was counted again on a second occasion six months later while being blinded to the initial result. 10 fields were counted using the same protocol as before. The values obtained (A.I.2) are described in the table below.

On Spearman rank correlation test the sets of apoptotic indices obtained on the two separate occasions (A.I.1 and A.I.2) were found to correlate very closely with $R_s = 0.9984$ and $p < 0.001$. This close correlation is also evident from the graph (Fig.5.1).

The final apoptotic index (A.I. Av) was calculated by averaging the two values obtained separately.. As can be seen from the table below these values are almost identical to the values obtained initially.

Table 5.1 Apoptotic indices of archival lung tumours as counted on two separate occasions and the average apoptotic indices.

	Minimum	Maximum	Mean	S.D	Median
A.I. 1	2.4	145.5	30.29	24.75	22.3
A.I. 2	2.8	146	30.28	24.74	22.45
A.I. Av.	2.6	145.7	30.17	24.78	22.4

The average apoptotic index has been used in all future analyses and these are the values that have been tabulated in Appendix C.

The frequency distribution of the apoptotic indices of these tumours is plotted in Fig.5.2. As can be seen the distribution is asymmetric with a suggestion of bimodality. It is clear from the graph that most cases have a relatively low rate of apoptosis—between 10-25/10,000 malignant cells. There is a second peak at 50 beyond which the numbers dwindle rapidly. To divide the population into high apoptosis and low apoptosis groups we used the upper quartile (41.2) as the cut-off point. By this definition there were 33 cases with a high apoptotic rate (i.e. $AI > 41.2$) and 101 cases with a low apoptotic rate.

Discussion

The staining method we used is widely available in every pathological laboratory and is easy to perform and much cheaper than immunohistochemical methods. The counting protocol was tedious but led to a highly reproducible means of quantifying apoptosis. In

retrospect, we need not have recounted all 134 slides as an analysis of a much smaller sample would easily have revealed the strong correlation. As it happened, we undertook statistical analysis only after all the slides had been counted twice. In the next section we have compared this protocol with another established method of quantifying apoptosis.

Although it was infrequent, there was some evidence of apoptosis in every lung tumour examined. The counts varied widely ranging from 3 to 145 per 10,000 malignant cells. The hint of bimodality in the distribution of the apoptotic indices suggests that the tumours could possibly belong to two distinct biological groups which have different propensities for apoptosis. This might be a reflection of the different underlying abnormalities of the apoptosis-regulating genes in different tumours. These possibilities are explored in many of the subsequent sections where we have tried to correlate apoptotic index with other clinical, pathological and genetic markers.

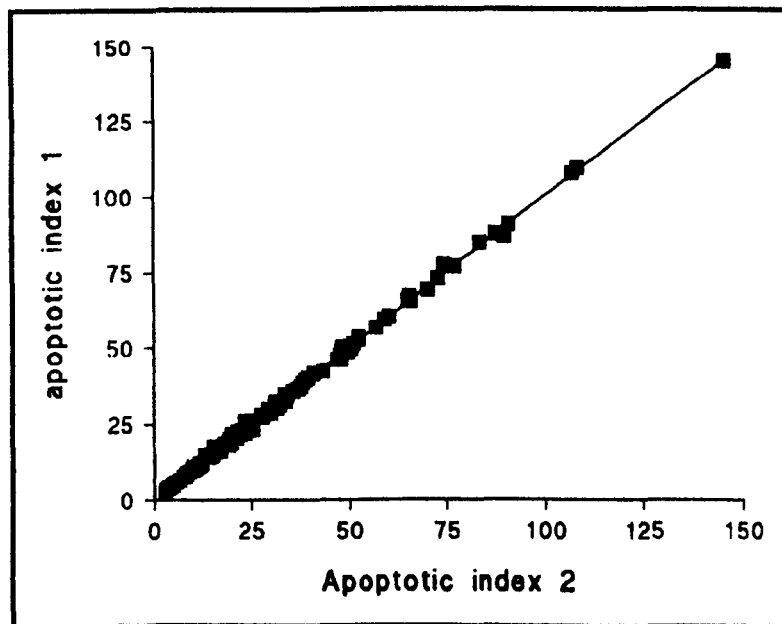


Fig. 5.1 Comparison of apoptotic indices of archival lung tumours as counted on two separate occasions.

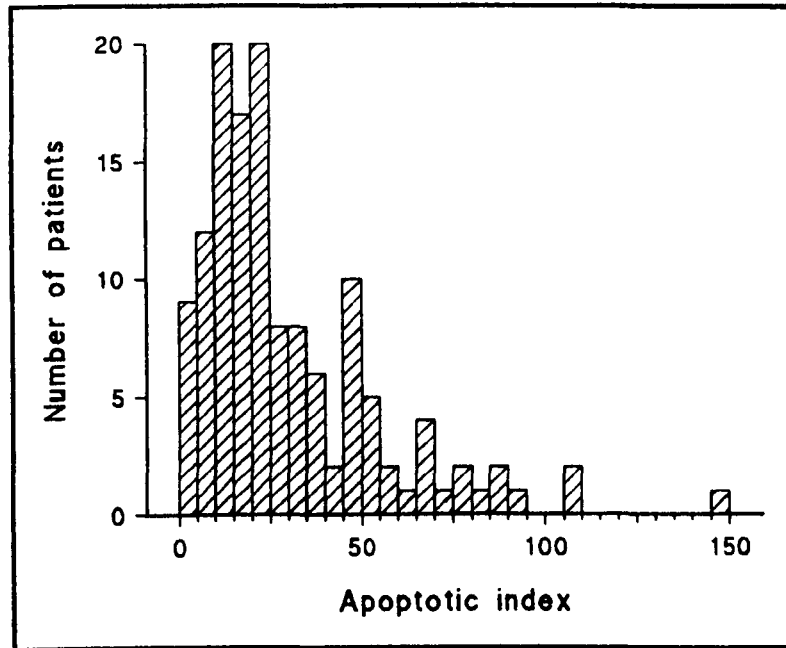


Fig. 5.2 Frequency distribution of apoptotic indices of the archival lung tumours.

5.2 Assessment of apoptosis by immunostaining with anti-ASP antibody.

Introduction

Our method of counting apoptosis in H&E stained sections led to very consistent results. However, the possibility remains that in counting stained apoptotic bodies, artefacts may lead to systematic under- or over-estimation of apoptotic indices. Thus it is important to employ a second method for measuring A.I. Of the available methods, discussed in Section 3.1, we chose to stain with the monoclonal antibody against Apoptosis Specific Protein or ASP (see Section 3.1.4.2).

ASP is a novel protein, structurally similar to the transcription factor AP-1, that is expressed at high levels in cells undergoing apoptosis but is not seen in viable cells or in cells dying by necrosis. However, as stated before in Section 3.1.4.2, some of these apoptotic marker proteins tend to be cell type specific and this antibody had not previously been tried on sections of lung tissue. Therefore, a primary aim of our investigation was to see if ASP was at all expressed in apoptotic cells originating from pulmonary epithelium. If it was found to be expressed it would serve as an excellent standard against which to compare our H&E results. The reliability of ASP staining has been shown in a recent comparative study on histological sections of Burkitt's Lymphoma, where staining with ASP led to the detection of more apoptotic cells than did ISEL or in situ nick labelling (Panchalingam *et al.*, 1996).

Results

We picked at random twenty-six slides from the archival series. We also chose to stain all the twenty fresh lung tumours that we had studied. These latter tumours included 11 squamous cell carcinomas, 6 adenocarcinomas, and one each of leiomyosarcoma, broncho-alveolar carcinoma and a case of undifferentiated NSCLC (see Sections 4.1.3 and

5.5.2). Staining was done with the c-jun/AP-1(Ab-2) antibody using standard immunohistochemical procedures as detailed in Section 4.2.4.

Good staining was seen in all forty six test slides. Apoptotic cells displayed a brown cytoplasmic staining in addition to the dark, condensed (and in many cases fragmented) nucleus (Fig. 5.3). Remains of apoptotic cells within phagosomes also took on a brown stain. These apoptotic cells stood out starkly amidst the malignant cells which had only taken up the light blue counterstain of Mayer's haemalum.

Apoptosis was quantified using the same protocol as for H&E. In brief, 10 oil immersion fields were chosen at random and the number of apoptotic and malignant cells in each field was counted. These slides were counted while being blinded to the result of the AI obtained from the corresponding H&E stained sections. The final apoptotic index was expressed as apoptotic cells/10,000 malignant cells.

Our study has proved that ASP is expressed in apoptotic pulmonary epithelium and therefore it is a valid way of assessing apoptosis in lung tissue.

The table below describes the apoptotic indices of these 46 tumours obtained by anti-ASP staining and H&E staining.

Table 5.2 Comparison of apoptotic indices using two different staining techniques (anti-ASP and H&E).

	Min	Max	Mean	S.D.	Median
A.I.(ASP)	6.6	159	60.7	39.8	50.9
A.I.(H&E)	4	90.8	36.7	24.2	27.3

When these two sets of values were subjected to Spearman rank correlation test they correlated very strongly with $R_s = 0.9760$ and $p < 0.001$. This close correlation is also evident from Fig. 5.4.

As stated above this group of tumours included 9 that were not SCCs. For the next part of the analysis we excluded these non SCC tumours. The apoptotic indices of this particular group are described in the next table.

Table 5.3 Comparison of apoptotic indices (SCCs only) using anti-ASP and H&E.

	Minimum	Maximum	Mean	S.D.	Median
A.I.(ASP)	13.9	159	62.18	38.97	51.3
A.I.(H&E)	9.2	90.8	37.18	23.17	27.6

On Spearman rank correlation test this second set of apoptotic indices correlated with each other just as strongly with $R_s = 0.9861$ and $p < 0.001$. Fig. 5.5 shows the regression graph for SCCs alone.

Discussion

Evidently, the two methods of assessing apoptosis in lung cancers correlate very strongly. This correlation is not confined to SCCs alone but holds true even when other histological types are included in the analysis.

In all the cases the count obtained by anti-ASP staining was higher than that obtained by H&E staining. This was not unexpected as ASP staining had clearly picked up cells in the very early and very late stages of apoptosis which would not have been identified by their morphological features alone. What is important for our purposes is that the two sets of values correlated so very strongly. This indicates that compared to ASP, H&E underestimated apoptosis by an almost fixed proportion in every case. Thus although the absolute value of apoptosis obtained by H&E would certainly be an under-estimate of the 'real' value, the indices are still valid for comparing the apoptotic states of different tumours.

However, there still remains the possibility that the apoptotic index as assessed in fixed sections by histological techniques is not a good reflection of the true apoptotic rate as this will be related to the rate of removal of the apoptotic bodies as well as to their rate of generation., and both these parameters may vary independently. The only way to measure

apoptotic rate is by serial estimation of apoptosis which of course is only possible in cultured lung tumour cells. We have attempted to do this by culturing cells obtained from malignant pleural effusions and freshly resected lung tumours. The results of these experiments will be presented in Section 5.5.

Having established a reliable index of apoptosis in 134 lung tumours, we moved on to determine whether this had any relevance to the biology and clinical behaviour of the tumours: particularly and primarily survival, but also other pathological parameters and markers related to apoptosis.

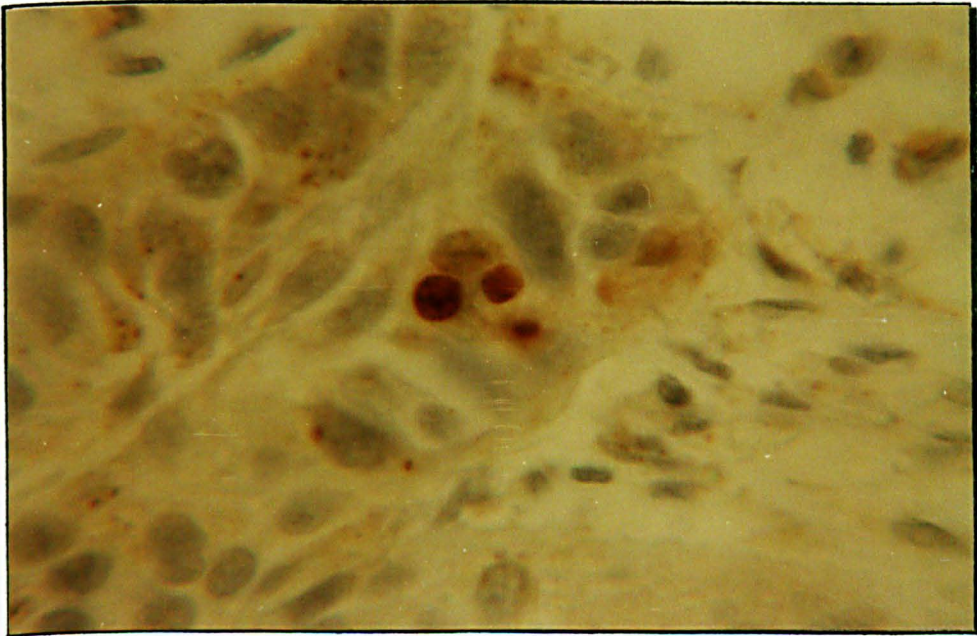


Fig. 5.3 Section of squamous cell lung cancer ($\times 630$) stained with monoclonal anti-ASP antibody. The cytoplasm of apoptotic cells have stained dark brown.

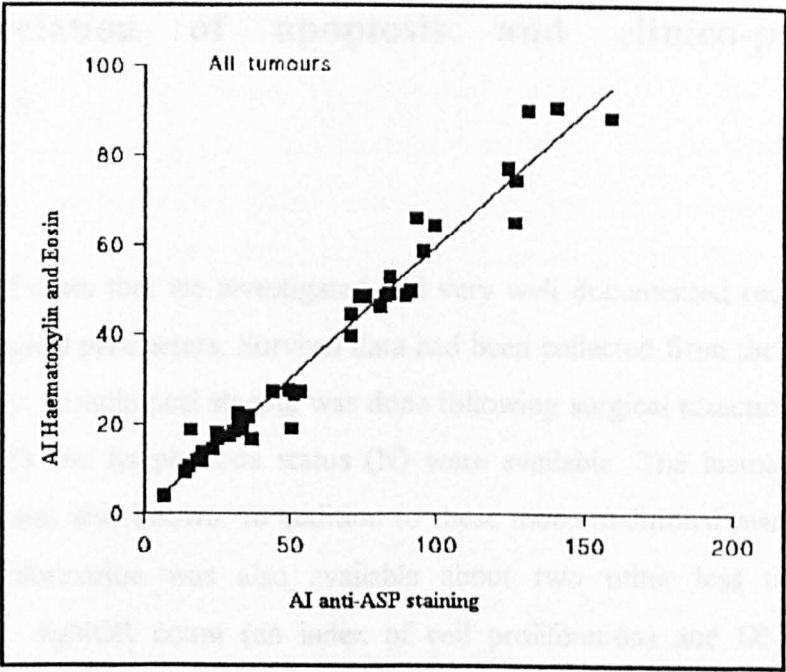


Fig.5.4 Comparison of apoptotic indices obtained by two different staining techniques (anti-ASP and H&E).

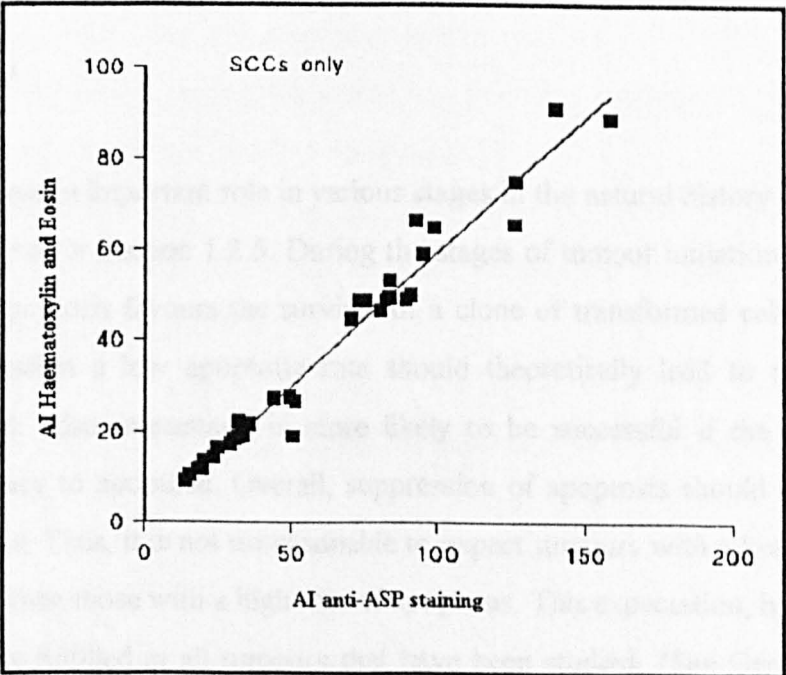


Fig. 5.5 Comparison of apoptotic indices (SCCs only) obtained by anti-ASP and H&E.

5.3 Correlation of apoptosis and clinico-pathological parameters

The series of cases that we investigated had very well documented records of several clinico-pathological parameters. Survival data had been collected from the West Midlands Cancer Registry. Pathological staging was done following surgical resection and details of tumour size (T) and lymph node status (N) were available. The histological grade of differentiation was also known. In addition to these more traditional markers of disease progression, information was also available about two other less commonly used parameters i.e. AgNOR count (an index of cell proliferation) and DNA ploidy. The availability of all this data gave us the opportunity to determine whether apoptosis was related to any of these.

5.3.1 Apoptosis and survival

Introduction

Apoptosis plays an important role in various stages in the natural history of a tumour, as discussed in detail in Section 1.2.5. During the stages of tumour initiation and promotion resistance to apoptosis favours the survival of a clone of transformed cells. Later during tumour progression a low apoptotic rate should theoretically lead to a faster rate of tumour growth. Also, metastasis is more likely to be successful if the cell has a low inherent tendency to apoptose. Overall, suppression of apoptosis should make a tumour more aggressive. Thus, it is not unreasonable to expect tumours with a low apoptotic rate to do less well than those with a high rate of apoptosis. This expectation, however, has not been universally fulfilled in all tumours that have been studied. (See General Discussion Section 6.3.)

We wanted to test this proposition in our series of cases. Survival data for this series had been collected by Boldy *et al.* (1991). They had contacted the West Midlands Cancer Registry to provide data on the clinical outcome, including cause of death from the death certificate, for all patients. Deaths in the first 30 days after surgery were regarded as post-operative deaths and were not included in the subsequent analysis. Patients were classified as having died as a result of their tumour if such a diagnosis was mentioned in part I of the death certificate.

Patients who were still alive at the last scheduled clinic follow up date at 10 years were assumed to have survived only up to that date and their survival was calculated accordingly. This assumption is certainly erroneous, but since follow up was terminated at 10 years this is the best that could be done under the circumstances.

Results

Of the 138 patients undergoing surgical resection 17 died in the first 30 days after surgery. Follow up was complete, apart from one patient who was lost to follow up after 110 months. 33 (27%) patients were alive at five years and 23 (17%) were alive at 10 years. Of the 99 deaths during the follow up period, carcinoma of the bronchus was reported as the main cause of death in 85 patients.

No correlation was seen between apoptotic index and survival. On Spearman rank correlation of the survival times and apoptotic indices of all patients (excluding post operative deaths), $R_s = -0.0096$ and $p = 0.911$.

We performed the same analysis excluding those cases who had died of causes other than lung cancer. Again no correlation was found, $R_s = -0.0199$ and $p = 0.834$. The graph is shown in Fig. 5.6.

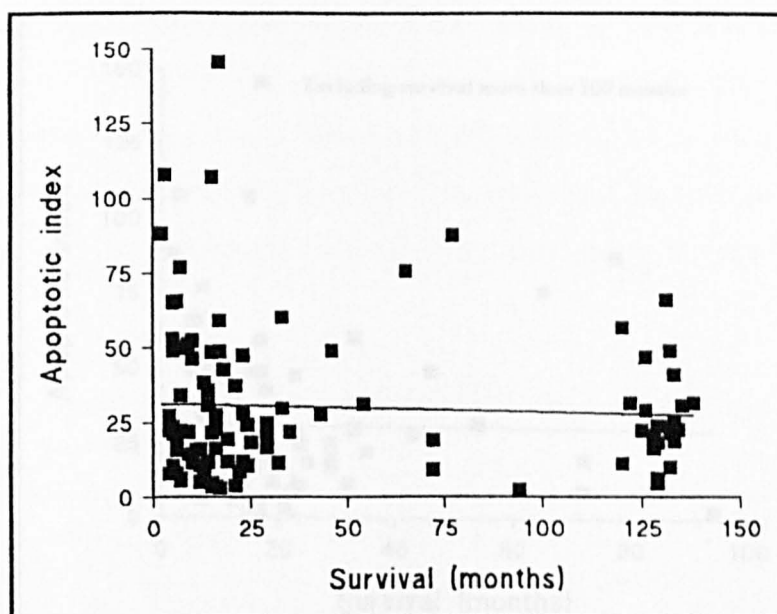


Fig 5.6 Correlation between apoptotic indices and survival times in squamous lung cancer.

Finally, we excluded patients whose survival was more than 100 months. They occupy one end of the graph in Fig. 5.6 above. These long lived patients might comprise a distinct biological group and we wanted to see how the rest of the group behaved in isolation. Also, by excluding this group we eliminated every patient whose survival time was in doubt. All but two members of this group had had a survival time *assigned* to them according to their date of last contact.

Even in this group there was no correlation between apoptosis and survival as shown in Fig. 5.7, $R_s = -0.0852$ and $p = 0.440$.

We next looked at survival times in groups with high and low apoptotic rates as defined in Section 5.1. In this and in all subsequent analyses of survival times, unless otherwise stated, we have excluded post-operative deaths and deaths due to causes other than lung cancer.

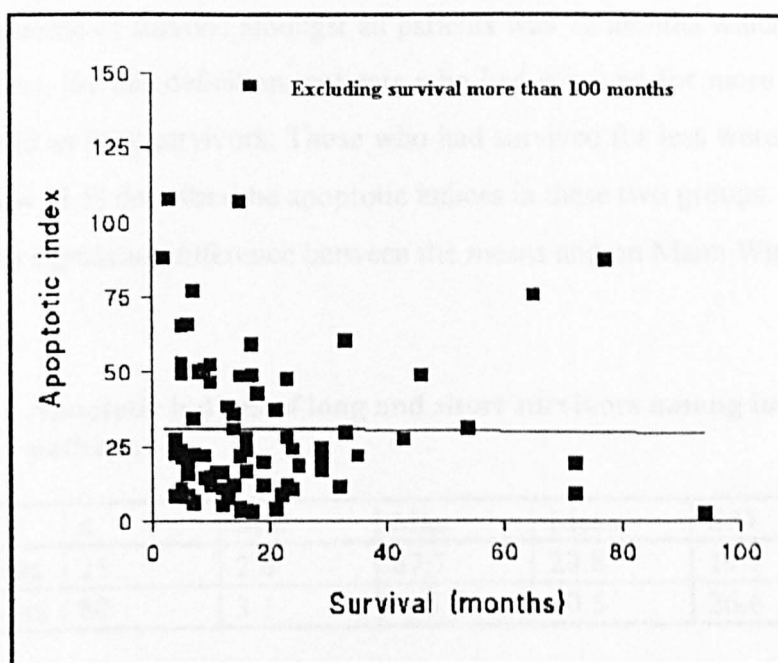


Fig. 5.7 Correlation between apoptotic indices and survival times in squamous lung cancer (excluding long survivors).

Survival times of patients in high apoptotic (i.e. AI >41.2) and low apoptotic groups are described in the table below (5.4).

Table 5.4 Survival times (in months) of squamous lung cancer patients with high or low apoptotic indices.

	n	Min	Max	Mean	S.D.	Median
High A.I.	27	2	132	34.4	43.3	15
Low A.I.	78	4	138	46.4	50	20

Although the mean survival in the low A.I. group was higher, on Mann Whitney U test this difference was not statistically significant, $p = 0.131$.

We then looked at the data from the opposite angle i.e. we divided the cases into long survival and short survival groups and analysed the apoptotic indices in each group.

The upper quartile of survival amongst all patients was 72 months which we assigned as the cut-off point. By this definition, patients who had survived for more than 72 months were considered as long survivors. Those who had survived for less were short survivors. The table below (5.5) describes the apoptotic indices in these two groups.

There was no significant difference between the means and on Mann Whitney U test, $p = 0.717$.

Table 5.5 Apoptotic indices of long and short survivors among lung cancer patients.

	n	Min	Max	Mean	S.D.	Median
Long survivors	25	2.6	87.7	28.8	19.9	23.6
Short survivors	80	3.1	145.7	30.5	26.6	22.2

Discussion

No matter which way we looked at it, A.I. could not be demonstrated to be a predictor of survival. The implications and the possible reasons behind this finding are discussed in detail later (see Section 6.3). Clearly, if it is accepted that apoptosis does influence the development of a tumour, there are other factors in individual cases which mitigate the effects of apoptosis. For instance disease stage, histological grade, proliferative state, oncogene expression—each could independently influence outcome and nullify any prognostic value of A.I. In our subsequent analyses we seek to determine whether these factors correlate with apoptosis and whether when we control for these factors, apoptosis correlated with survival. Although this is a dubious procedure because of small numbers in each subgroup, and potentially could generate significant correlations by chance, we felt that as we had a large group of cases it was worth the attempt.

5.3.2 Apoptosis and Disease Stage

Introduction

Disease stage, which is a composite index of tumour size, nodal metastasis and distant metastasis is one of the most important prognostic indicators in cancer. In this present series of cases pathological TNM staging was undertaken independently by two of the original investigators who reviewed all the information available from the hospital case notes. The UICC classification was used (see Appendix B) and patients were allocated in one of five pathological stages - I, II, IIIa, IIIb and IV. Where there was disagreement, all available data were re-examined for a consensus opinion.

Boldy *et al.* found lower disease stage to be a strong indicator of good prognosis in this series. We investigated if the apoptotic index bore any relation to the disease stage. Also we investigated if apoptosis and survival correlated within each disease stage

Results

The apoptotic indices of patients in each of the five pathological stages are described in Table 5.6.

Table 5.6 Apoptotic indices of patients in each pathological stage.

	n	Min	Max	Mean	S.D.	Median
Stage I	45	4.9	87.7	30.8	18.9	25.6
Stage II	47	3.8	107.2	24.7	22.1	18.3
Stage IIIa	27	3.1	108.7	35.2	28.6	22.7
Stage IIIb	11	2.6	145.7	38	40.7	30.8
Stage IV	4	4.7	69.7	30.5	27.9	23.9

On Mann Whitney U test there was no significant difference between the means in the five groups. Thus we concluded that apoptosis does not correlate with disease stage.

We next divided patients according to their disease stage and looked for correlation between apoptosis and survival within each stage by Spearman rank correlation test. Table 5.7 below describes the survival times of patients in each stage. The last two columns describe the coefficients of correlation between A.I and survival. As stated before, when assessing survival, patients who died in the immediate post-operative period and patients who died later of causes other than lung cancer were excluded from the analysis. Thus the number of entries in this table is less than in the table above. This discrepancy will be noticed in subsequent pairs of tables as well.

Table 5.7 Survival times of patients in each pathological stage and correlation between AI and survival in each stage.

	n	Min	Max	Mean	S.D.	Median	Rs	p
Stage I	34	4	133	57.6	52.5	30.5	-0.0639	0.716
Stage II	39	4	138	42.1	49	16	0.1474	0.373
Stage IIIa	20	2	134	30.3	39.7	14.5	-0.2635	0.259
Stage IIIb	11	5	135	30	42.9	15	-0.0455	0.883

There was only one patient in Stage IV who survived beyond a month. He lived for 15 months and had an A.I. of 4.7.

As the coefficients of correlation in the table indicate no correlation could be demonstrated within any disease stage.

Discussion

This series is not truly representative of a group of patients with lung cancer. The higher disease stages have been grossly under-represented here because they would not have been offered surgical resection as treatment. For instance only 10.9% of patients in this series were in Stages IIIb and IV whereas in actual fact when patients first present with lung cancer, about 70% of them will be in these stages. Also the actual numbers involved in the two higher stages are quite low. Therefore, from these results, it is difficult to draw any definite conclusions about the relevance of apoptosis in high disease

stage. However even among the lower disease stages—I, II, IIIa—no trend is observed in the apoptotic indices and no correlation is seen between A.I. and survival.

5.3.3 Apoptosis and Lymph Node Metastasis

Introduction

Having failed to discern any correlation between apoptosis and disease stage, we next planned to look more closely at one particular determinant of stage i.e. metastasis. This present series comprised patients who had been chosen for surgical treatment and those with distant metastases were largely excluded. Yet there were four patients in this series who despite metastases had undergone surgical resection but the numbers were too low to draw any meaningful conclusions. We therefore concentrated only on lymph node metastasis. Patients could be divided into three groups - N0, N1 & N2 - according to the degree of lymphatic spread (see Appendix B) and we wanted to see if the apoptotic index bore any relation to this.

Reduced susceptibility to apoptosis should make it easier for a cancer cell to survive in the alien environment of a metastatic organ where the lack of many of the usual growth factors is likely to push the cell towards apoptosis (see Section 1.2.5.3). Indeed metastatic prostatic cancer cells have been shown to be less prone to apoptosis. Thus theoretically one could expect to see an inverse correlation between apoptosis and lymph node spread.

Results

The apoptotic indices of patients within the three groups are described in Table 5.8 below.

Table 5.8 Apoptotic indices of patients grouped according to lymph node status.

	n	Min	Max	Mean	SD	Median
N0	54	4.7	87.7	30.4	19.3	25.1
N1	62	2.6	145.7	27.9	26.9	18.8
N2	16	3.1	108.7	35.5	29	22.3

On Mann Whitney U test there was no significant difference between these three means. Thus the apoptotic rate of a tumour does not seem to influence its ability to metastasise to lymph nodes.

Discussion

Our findings do not conclusively rule out the possibility of a link between apoptosis and lymph node metastasis. From the main tumour a sub-clone of cells with a lower apoptotic rate (among other changes) might have been selected out to colonise lymph nodes. This could have been apparent if we had checked the apoptotic rate in the metastatic lymph node rather than in the parent tumour.

5.3.4 Apoptosis and histological differentiation

Introduction

It was once generally believed that less well differentiated tumours were more aggressive and more metastatic than more differentiated tumours. It is now appreciated that this is an oversimplification and, in fact, not a very accurate way to assess the degree

of malignancy for all kinds of tumours. However, for certain epithelial tumours, such as carcinoma of the cervix, uterine endometrium, colon, and thyroid, histologic grading is a fairly accurate index of malignancy and prognosis.

In their study Boldy *et al.* graded the tumours in their series into three groups according to the degree of histological differentiation. They found no significant difference in survival between well differentiated, moderately differentiated and poorly differentiated tumours.

In our own study we have investigated if any correlation exists between the degree of differentiation and the amount of apoptosis in a tumour.

Results

It was possible to grade 114 tumours according to their degree of differentiation. The apoptotic indices for the three groups are described in Table 5.9.

Table 5.9 Apoptotic indices of patients in each grade of histological differentiation.

	n	Min	Max	Mean	S.D.	Median
Well diff	12	6.5	75.8	27.6	21.1	21.3
Moderate diff	58	3.1	108.7	28.4	23.5	21
Poor diff	44	2.6	96.8	29.5	22.3	23

On Mann Whitney U test there was no significant difference between the means.

We next investigated if apoptosis correlates with survival within any of these 3 groups. Table 5.10 below describes the survival times of patients within each group of histological differentiation. The last two columns indicate the coefficients of correlation (Spearman rank) between apoptosis and survival.

Table 5.10 Survival times of patients in each grade of differentiation and correlation between AI and survival within each group.

	n	Min	Max	Mean	S.D.	Median	Rs	p
Well diff	9	5	135	60.5	56.5	29	-0.3333	>0.05
Mod diff	49	2	132	30.5	38.6	15	0.0367	0.810
Poor diff	33	4	134	48.3	52.3	19	0.0307	0.869

The high p values indicate the lack of correlation within any group.

Conclusion

No correlation exists between apoptotic index, the degree of histological differentiation and survival in this series of tumours.

5.3.5 Apoptosis and AgNOR

Introduction

Measurement of the AgNOR count of a histological section is a convenient and reliable way off assessing the proliferative state of a tissue.

The nucleolus is composed of structural and functional units called nucleolar organizer region (NOR). Each NOR contains a cluster of ribosomal ribonucleic acid (rRNA) genes and the enzymes and proteins necessary to transcribe these genes and pack the products into ribosomes. Many of the associated proteins within a NOR have a very high affinity for silver –a property that is utilised in staining them (Ploton *et al.*, 1986). The method involves staining dewaxed 3µm sections with a 1:2 mixture of gelatin and formic acid and 50% aqueous silver nitrate. On silver staining of histological sections the NORs appear as dark black dots called AgNOR which can be easily identified and counted.

The AgNOR count of a cell depends on its proliferative state. Resting cells have been found to have only one NOR. In proliferating cells the need for increased ribosomal biogenesis is met by increased rRNA transcriptional activity within nucleoli.

Morphologically this is reflected by an increased number of NORs and therefore a high AgNOR count. A large number of studies have proved that the AgNOR count of a tissue correlates very closely with its rate of proliferation. These studies have been done on a wide range of tissues where AgNOR has been compared to other reliable measures of cell proliferation e.g. Ki-67 immunostaining, bromodeoxyuridine (BrdU) labelling and S-phase estimation on DNA flow cytometry. A very strong correlation was seen in all but a few instances. The main advantage of AgNOR over all these other methods is that it can be done on paraffin embedded, routinely processed histological sections and there is no need for fresh tissue (flow cytometry) or frozen sections (Ki-67). A high AgNOR count has been found to adversely affect prognosis in a wide range of malignant conditions (Derenzini and Ploton, 1994: for a review of studies demonstrating correlation between AgNOR quantity and cell proliferation and of studies demonstrating the prognostic relevance of interphase AgNOR quantity in tumour sections).

Boldy *et al.* were able to obtain good AgNOR staining of histological sections in all but 10 of the 138 cases of this series by using the method mentioned above. All the tumours had significantly higher AgNOR counts than some areas of normal pseudostratified columnar epithelium that were also examined for comparison. AgNOR scores were not related to survival in the group as a whole. Nor was there any correlation with survival when patients were grouped according to disease stage. Although AgNOR numbers tended to be higher in the less well differentiated tumours the range of AgNOR scores in each histological subgroup was wide and there was considerable overlap between the groups. There was no correlation between AgNOR count and DNA ploidy.

Combining our own investigations with these results obtained by Boldy *et al.* we wanted to investigate if any correlation exists between the AgNOR count and the apoptotic index of a tumour. Seeing that the same genes are often involved in the control of both cell proliferation and apoptotic cell death it is not entirely illogical to expect some degree of reciprocal relationship between these two indices.

Results

AgNOR counts could be obtained in 104 cases. The counts ranged from 4.6 to 24 per cell nucleus per specimen. Mean AgNOR was 11.3 and S.D. 3.9. Spearman rank correlation test between AgNOR and the corresponding AIs showed no correlation with $R_s = 0.1070$ and $p = 0.280$. The graph displaying this lack of correlation is shown in Fig. 5.8.

For the purpose of analysis Boldy *et al.* had divided the tumours into four groups according to the AgNOR counts.

Group I	AgNOR <8
Group II	AgNOR 8--<12
Group III	AgNOR 12--<16
Group IV	AgNOR 16+

We looked for a correlation between A.I. and survival within each of these four groups. Table 5.11 describes the survival times in each group. The last two columns describe the coefficients of correlation (Spearman rank correlation) between A.I. and survival.

Table 5.11 Survival times of patients in each AgNOR group and correlation between AI and survival within each group.

	n	Min	Max	Mean	S.D.	Median	R_s	p
Gr. I	14	5	134	50.6	54.7	15.5	0.1011	0.736
Gr. II	37	4	135	36.0	41.4	17	-0.0065	0.966
Gr. III	21	3	133	35.3	46.9	16	-0.0951	0.678
Gr. IV	9	5	132	52.7	57.3	19	0.1333	>0.05

Conclusion

Thus we concluded that apoptosis and AgNOR counts are not related in these tumours. In other words there is no correlation between cell death and cell proliferation.

Although apoptosis or cell proliferation taken each in isolation might not predict prognosis, reason would persuade us that the two taken together might have a better chance of correlating with outcome. Our analysis proves that this does not hold true for squamous lung cancers.

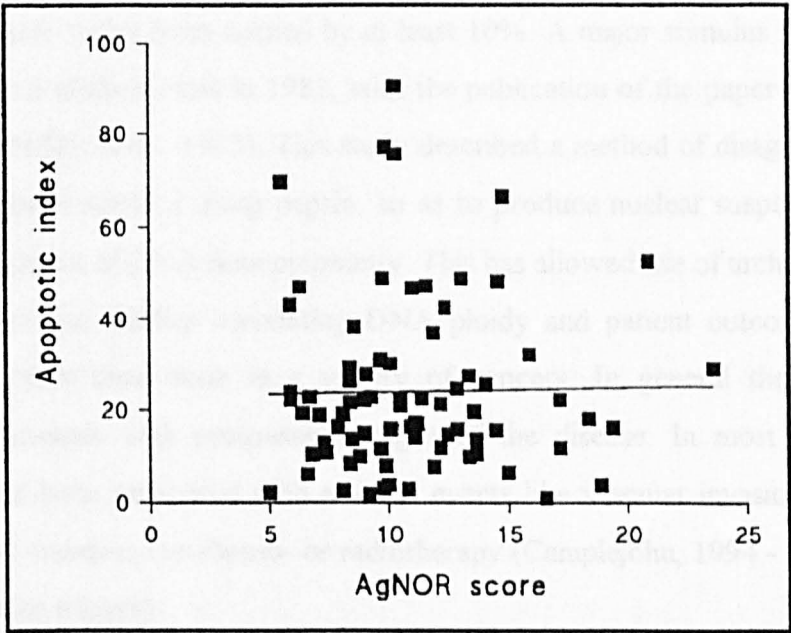


Fig. 5.8 Correlation between AI and AgNOR counts in squamous lung cancer.

5.3.6 Apoptosis and DNA ploidy

Introduction

Aneuploidy is considered to be a marker of aggressive tumour behaviour. The genetic instability manifest during tumour progression is characterised by a variety of aberrations in the genome. While the more subtle changes—point mutations, gene

deletions and rearrangements—may be associated with the initiation of the transformation process, gross changes in the number of chromosomes usually occur as tumours progress in malignancy. Loss or duplication of chromosome segments or of whole chromosomes is common in these late stages and is often associated with the acquisition of a more aggressive phenotype (see Section 1.1.4.5).

DNA flow cytometry is one way of assessing such changes in a cell's total DNA content. It is a relatively crude way because abnormalities become detectable only when the DNA content varies from normal by at least 10%. A major stimulus to clinical flow cytometric DNA studies came in 1983, with the publication of the paper by Hedley and co-workers (Hedley *et al.*, 1983). This study described a method of disaggregating thick paraffin embedded sections using pepsin, so as to produce nuclear suspensions suitable for the performance of DNA flow cytometry. This has allowed use of archival material to set up retrospective studies correlating DNA ploidy and patient outcome. Numerous such studies have been done in a variety of cancers. In general the frequency of aneuploidy increases with progressive stages of the disease. In most cancers DNA aneuploidy has been associated with adverse events like vascular invasion, lymph node metastasis and resistance to chemo- or radiotherapy (Camplejohn, 1994 - for a review of literature on this subject).

In lung cancers controversy exists regarding the utility of tumour DNA content in predicting clinical outcome. While some studies have linked aneuploidy to decreased survival when compared to diploid tumours of similar stage (e. g. Isobe *et al.*, 1990; Zimmerman *et al.*, 1987), others have been unable to confirm such a correlation (e.g. Carp *et al.*, 1992; TenVelde *et al.*, 1988).

Boldy *et al.* prepared samples from 60µm paraffin sections by the method described by Hedley and colleagues. The nuclei were stained with propidium iodide and analysed flow cytometrically. Tumours were classified as DNA aneuploid if a discernible peak was visible that was separate from the main G0/G1 peak of normal cells or if the apparent G2M peak channel:G0/G1 peak channel ratio was greater than 1.1 or less than 0.9. In this particular series DNA ploidy did not correlate with survival.

Drawing on this data we have tried to find out if apoptosis correlates with ploidy in squamous cell carcinoma of lung.

Results

Ploidy data could be obtained in 107 cases. Of these 44 were diploid and 63 aneuploid. No satisfactory DNA histogram could be obtained for the others. The apoptotic indices for the diploid and aneuploid tumours are described in the table 5.12.

Table 5.12 Apoptotic indices of diploid and aneuploid tumours.

	n	Min	Max	Mean	S.D.	Median
Diploid	44	3.1	107.2	26.3	23.2	20.8
Aneuploid	63	2.6	108.7	29	20.9	22.4

On Mann Whitney U test there was no significant difference between the means, $p = 0.241$.

We next investigated if apoptosis correlated with survival within each of these groups. The table below describes the survival times of patients who were diploid or aneuploid. The last two columns describe the coefficients of correlation (Spearman rank correlation) between apoptosis and survival.

Table 5.13 Survival times of patients with diploid and aneuploid tumours and correlation between AI and survival within each group.

	n	Min	Max	Mean	S.D.	Median	R_s	p
Diploid	39	2	135	42	49.4	16	-0.0444	0.780
Aneuploid	49	3	133	38	43.5	18	-0.0811	0.575

Thus, even when the variable of tumour ploidy was eliminated, apoptosis did not correlate with survival.

Conclusion

There is no correlation between apoptotic index, DNA ploidy and survival in this series of tumours.

5.4 Immunohistochemical staining of lung cancer

Having comprehensively analysed the interrelationship of apoptosis and the more traditional pathological markers, we shall now move on to examine directly some of the proteins known to be important in apoptosis. There are many potential targets for investigation, but given that we were analysing 134 tumours we needed to be very selective. We have chosen two proteins. One of them is Bcl2, a key player in the control of apoptosis in most cells. The other is CD40 - a cell surface protein known to protect B lymphocytes from apoptosis, although its exact role in epithelial cells has not been fully defined yet. CD40 activation has been shown to cause upregulation of bcl2 in some cells, so the function of the two proteins may be inter-linked. For each protein we first analysed its expression among the series of lung tumours. We then proceeded to examine for correlation between gene over-expression and other clinico-pathological parameters.

5.4.1 Expression of Bcl2 in squamous cell carcinoma of lung

Introduction

bcl2 is one of the key apoptosis regulating genes (see Section 1.3). Over-expression of Bcl2 protein renders a cell fairly resistant to apoptosis without increasing the rate of proliferation. Activation of this oncogene with consequent over-expression of the protein is seen in a wide variety of lymphoid and non-lymphoid tumours. Bcl2 over-expression has been reported in several series of non small cell lung cancers (see Section 6.5).

We investigated the expression of Bcl2 in our series of 134 cases of squamous cell carcinoma of lung. We wanted to see what fraction of squamous cell carcinomas displayed activation of this oncogene. We also attempted to quantify the strength of this activation in each tumour separately by counting the percentage of Bcl2 +ve malignant cells.

Method

Paraffin sections were stained with monoclonal antibody to Bcl2. The method is described in detail in Section 4.2.2. The stained sections were examined under the microscope using an oil-immersion lens producing a magnification of $\times 1000$. Bcl2 positive cells appeared brown (Fig. 5.9). This stain was distributed predominantly in the cytoplasm of the positive cells. Infiltrating lymphocytes in the fibrous stroma often stained positive for Bcl2 and served as an internal positive control for the staining method.

The staining was heterogeneous. In many tissue sections there were both areas of strongly stained cells and completely unstained cells in varying proportions (Fig. 5.10). There were also instances where all the malignant cells had behaved identically i.e. they had all taken up the stain or had all remained unstained. We assumed that such differences in staining within a section was a reflection of the strength or degree of bcl2 activation. We quantified this by calculating a Bcl2 index for each tumour.

Ten fields were chosen at random from different parts of the tumour section. The number of stained and unstained malignant cells in each field was recorded. The final percentage of stained malignant cells in all ten fields was expressed as Bcl2 index i.e. number of Bcl2 +ve malignant cells/100 malignant cells. The values for individual tumours have been tabulated in Appendix C.

Results

In all 75 cases (55.6%) expressed Bcl2 in at least some of their cells while 59 cases (44.4%) showed no expression of this protein whatsoever.

Of the tumours that did express this protein the Bcl2 index ranged from 1.4 to 100 with a mean of 54.4 (S.D. 39.1). When the Bcl2 indices were charted in a frequency distribution graph (Fig. 5.11) a bi-modal distribution was clearly evident. A sizeable number of tumours had values at the two extremes while there were very few if any

tumours with a Bcl2 index in the range 40-50. Thus for this series a Bcl2 index of 50 or above could be taken as a cut off point to determine if a tumour was Bcl2 positive or Bcl2 negative.

By this definition 42 tumours (31.6%) in this series were Bcl2 positive and 92 tumours (68.4%) were Bcl2 negative.

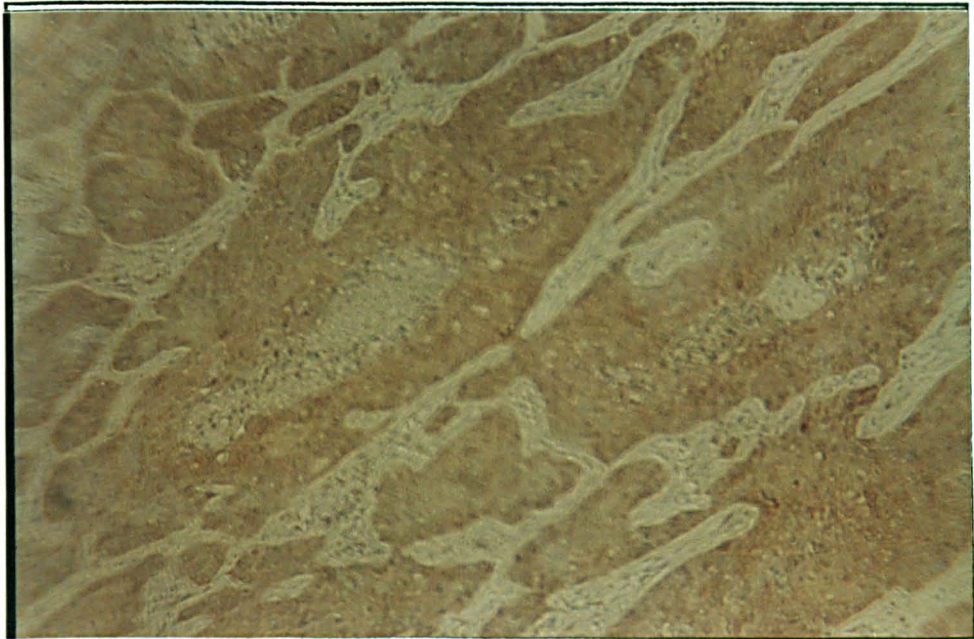


Fig. 5.9 Section of squamous cell lung cancer ($\times 125$) stained with monoclonal antibody to Bcl2. The tumour cells have stained uniformly brown while the stromal tissue has remained unstained.

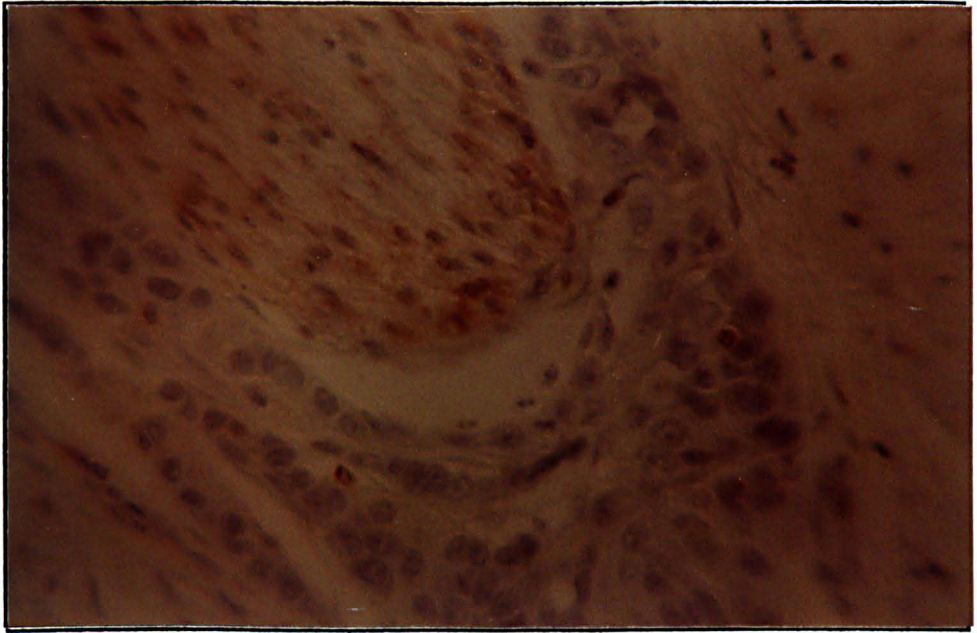


Fig. 5.10 Section of squamous cell lung cancer ($\times 630$) stained with monoclonal antibody to Bcl2. Tumour cells at the top half of the picture have stained positive (brown). Adjacent tumour cells in the lower half of the picture are unstained (blue). Infiltrating lymphocytes have stained positive.

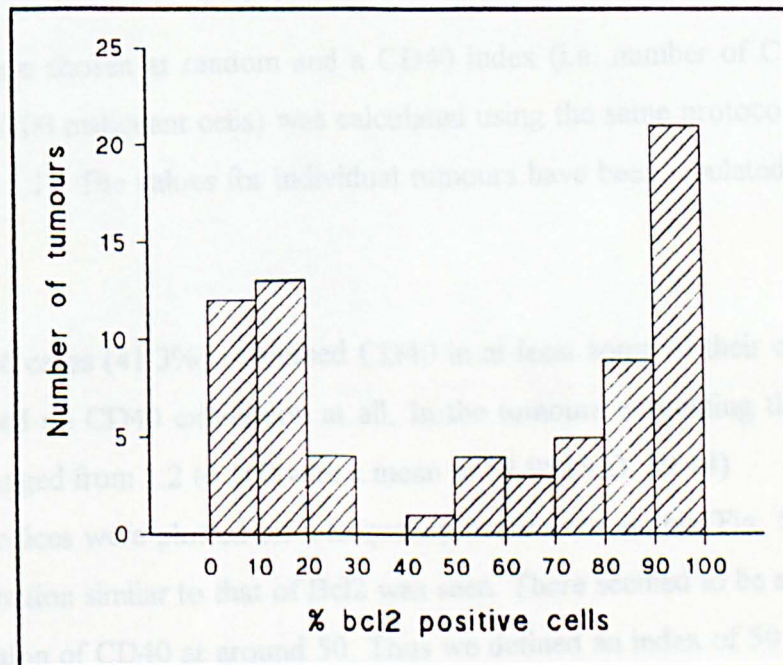


Fig. 5.11 Frequency distribution of Bcl2 indices in squamous lung cancer.

5.4.2 Expression of CD40 in squamous cell carcinoma of lung

We next examined the expression of CD40 in our series of cases. As in the case of Bcl2, we quantified the number of CD40 +ve malignant cells in each tumour to obtain a CD40 index.

Results

Paraffin sections were stained with monoclonal antibody to CD40 by a method detailed in Section 4.2.3. These stained sections were examined under the oil immersion lens (magnification $\times 1000$). In CD40 positive cells brown staining was seen on the cell surface as well as in the cytoplasm. CD40 negative cells appeared faint blue due to the counter-stain of Mayer's Haemalum. Many lymphocytes as well as parts of the fibrous stroma were seen to stain positively with CD40 as well (Fig. 5.12).

As in the case of Bcl2 many sections contained a variable mixture of stained and unstained malignant cells whereas others were uniformly positive or negative. We calculated a CD40 index for each tumour based on the percentage of positively staining cells.

Ten fields were chosen at random and a CD40 index (i.e. number of CD40 positive malignant cells/100 malignant cells) was calculated using the same protocol as for Bcl2. (See Section 5.5.1). The values for individual tumours have been tabulated in Appendix C.

Altogether 56 cases (41.3%) expressed CD40 in at least some of their cells. 78 cases (58.7%) showed no CD40 expression at all. In the tumours expressing the antigen the CD40 index ranged from 1.2 to 100 with a mean of 53.95 (S.D. 39.44)

The CD40 indices were plotted on a frequency distribution graph (Fig. 5.13). Again a bimodal distribution similar to that of Bcl2 was seen. There seemed to be a natural break in the distribution of CD40 at around 50. Thus we defined an index of 50 to be the cut-

off point above which a tumour would be deemed to be CD40 positive. By this definition 29 tumours (21.8%) in this series were CD40 positive and 105 tumours (78.2%) were CD40 negative.

In subsequent sections we have investigated if Bcl2 or CD40 expression correlates with apoptosis, survival or any other clinical or pathological parameter. But first, we looked for cross correlation between Bcl2 and CD40.

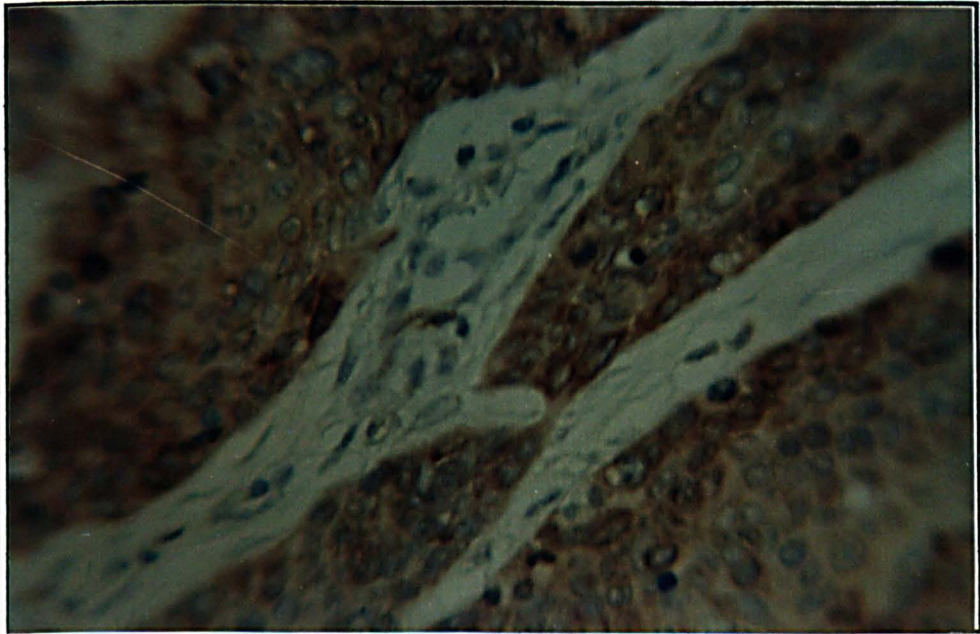


Fig. 5.12 Section of squamous cell lung cancer (×250) stained with monoclonal antibody to CD40. The stromal elements are starkly negative.

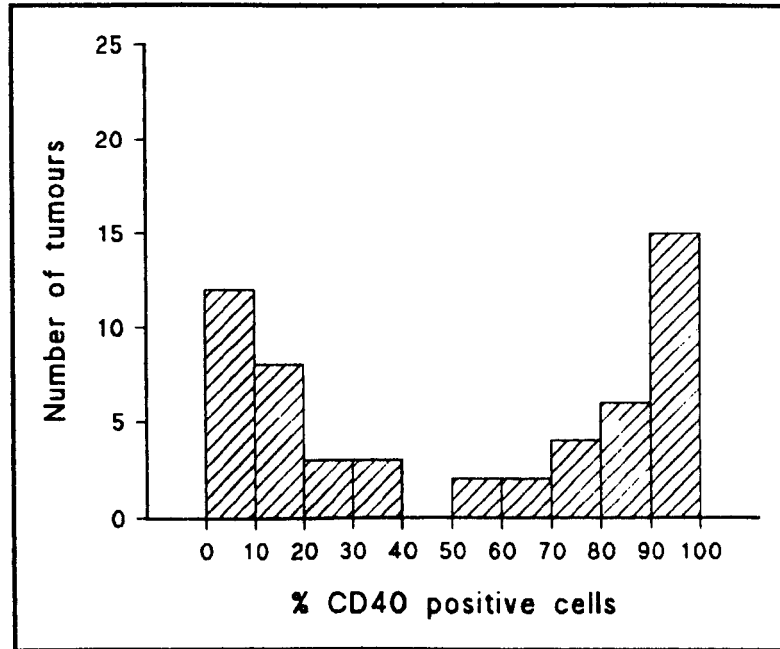


Fig. 5.13 Frequency distribution of CD40 indices in squamous lung cancer.

5.4.3 Cross correlation of Bcl2 and CD40 expression in squamous cell carcinoma of lung

In epithelial cells and carcinomas the relation between Bcl2 and CD40 has not been elucidated yet (see Section 1.4.5.3). In our series of lung cancers we examined if the expression of one oncogene influenced the expression of the other. Cases were divided

into four groups according to their Bcl2 and CD40 status as defined in the previous two chapters. This grid-table shows the number of cases in each of the four groups.

Table 5.14 Lung tumours grouped according to Bcl2 and CD40 status.

	Bcl2 +ve	Bcl2 -ve
CD40 +ve	6	23
CD40 -ve	36	69

Chi-square test was performed on these values.

Chi square = 1.952. Degree of Freedom = 1. Yates correction = 1.371.

The tabulated value for one degree of freedom at 95% probability is 3.841. Therefore the distribution is not significantly skewed.

Next we performed a Spearman rank correlation test on the Bcl2 and CD40 indices. There was a tendency towards inverse correlation between the two ($R_s = 0.005$ and $p = 0.064$) which is also evident from the graph shown in Fig. 5.14. When Kendall's rank correlation was done on the same set of values, $p = 0.057$. However, when the zero values of Bcl2 and CD40 were excluded there was no correlation on Spearman rank test, $R_s = 0.2141$ and $p = 0.300$ (see Fig. 5.15).

Conclusion

Thus, Bcl2 and CD40 expression in lung tumours tend to correlate inversely when considered as a continuous variable, but not when considered as a dichotomous variable (i.e. positive or negative).

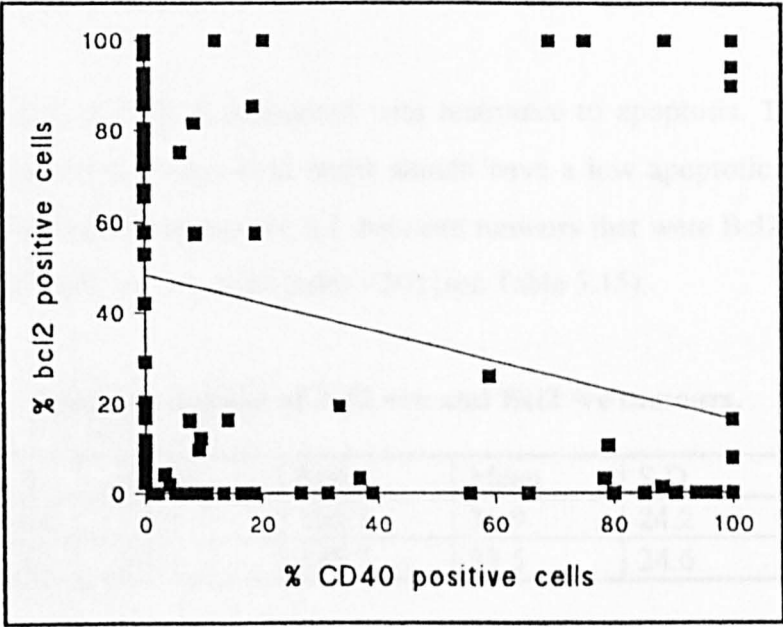


Fig. 5.14 Correlation between Bcl2 and CD40 indices in squamous lung cancer.

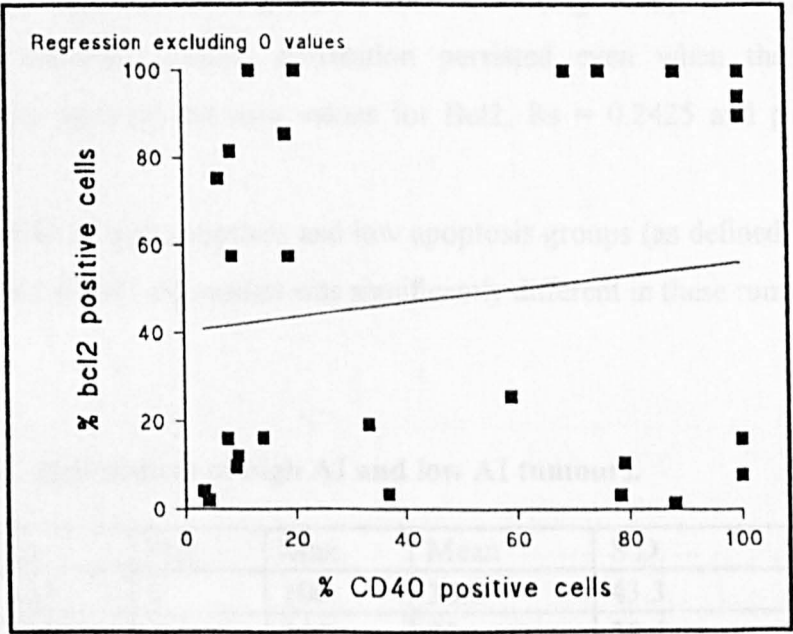


Fig. 5.15 Correlation between Bcl2 and CD40 indices in squamous lung cancer (excluding zero values).

5.4.4 Bcl2 and apoptosis

Over-expression of Bcl2 is associated with resistance to apoptosis. Thus one would expect that cases with a high Bcl2 index should have a low apoptotic index and vice versa. We looked for differences in A.I. between tumours that were Bcl2 +ve (i.e. Bcl2 Index >50) and Bcl2 -ve (i.e. Bcl2 Index <50) (see Table 5.15).

Table 5.15 Apoptotic indices of Bcl2 +ve and Bcl2 -ve tumours.

	n	Min	Max	Mean	S.D.	Median
Bcl2 +ve	42	2.6	108.7	35.9	24.2	28.5
Bcl2 -ve	92	3.1	145.7	27.5	24.6	20.3

On Mann Whitney U test the difference between the means was significantly different, $p = 0.008$.

We then performed Spearman rank correlation test between B.I. and A.I. to ensure that the correlation demonstrated above was not an effect of the way we had defined Bcl2 positivity. The values showed significant correlation (Fig. 5.16), $R_s = 0.2135$ and $p = 0.041$. This tendency towards correlation persisted even when the analysis was performed after ignoring the zero values for Bcl2, $R_s = 0.2425$ and $p = 0.041$ (Fig. 5.17).

Next we looked at high apoptosis and low apoptosis groups (as defined in Section 5.1) and investigated if Bcl2 expression was significantly different in these tumours (see Table 5.16).

Table 5.16 Bcl2 indices of high AI and low AI tumours.

	n	Min	Max	Mean	S.D.	Median
High AI	33	0	100	36.6	43.3	12.2
Low AI	101	0	100	28	38.4	3.6

Though the High AI group had a higher mean Bcl2 index the on Mann Whitney U test the difference was not statistically significant, $p = 0.824$.

Conclusion

Although it was not evident on every method of analysis, on the whole, tumours that expressed a high level of Bcl2 also had a high rate of apoptosis but the statistical significance was not always very strong. This is contrary to our expectations based on the known biological role of Bcl2. We are not immediately able to explain this finding. Eerola *et al.* in their study involving 40 small cell lung cancers have recently described a similar positive correlation between bcl2 expression and apoptosis (Eerola *et al.*, 1997). It may be that the concomitant expression of other Bcl2 family members holds the key to this enigma. (see Section 6.6)

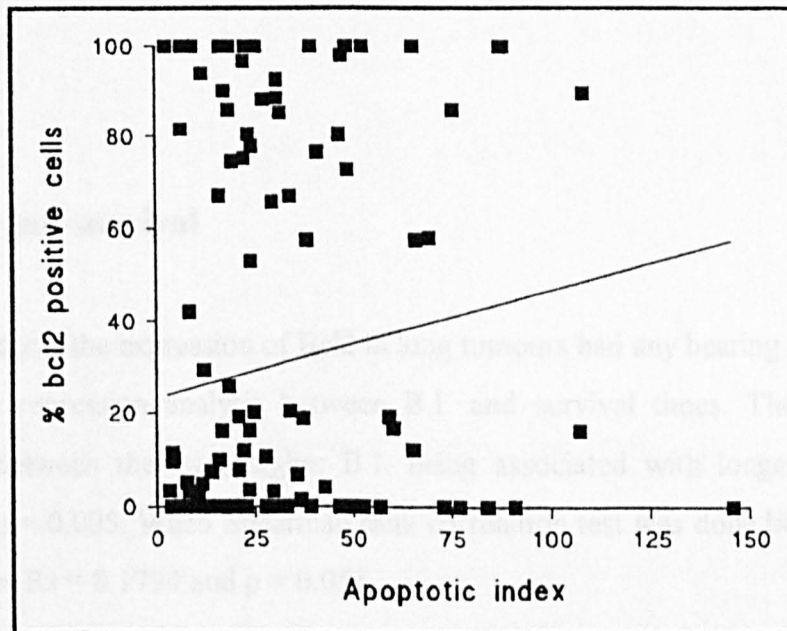


Fig. 5.16

Correlation of Bcl2 index and apoptotic index of lung tumours.

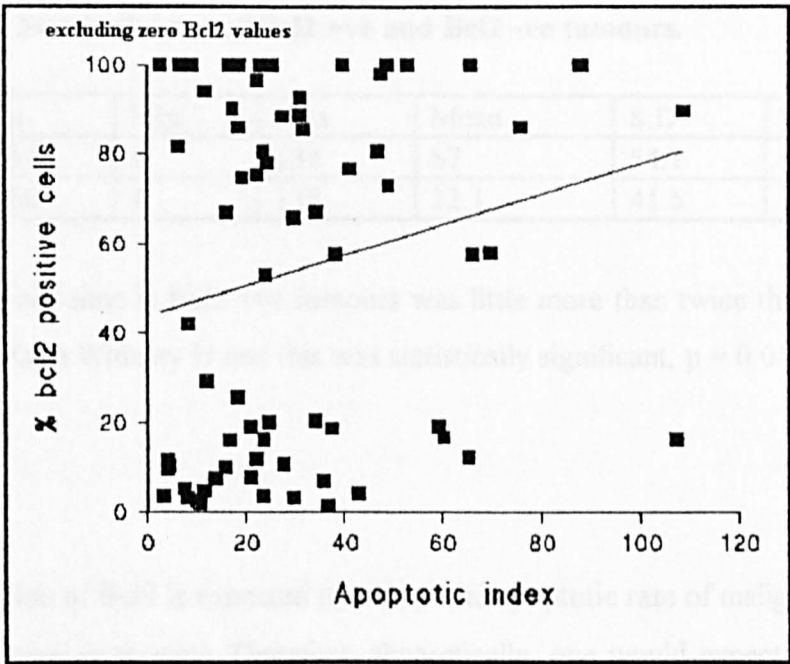


Fig. 5.17 Correlation of Bcl2 index and apoptotic index of lung tumours (excluding zero Bcl2 values).

5.4.5 Bcl2 and survival

To investigate if the expression of Bcl2 in lung tumours had any bearing on survival, we performed a regression analysis between B.I. and survival times. There was strong correlation between the two, higher B.I. being associated with longer survival, $r = 0.2720$ and $p = 0.005$. When Spearman rank correlation test was done between B.I. and survival times $R_s = 0.1794$ and $p = 0.053$.

We next investigated if the survival times in Bcl2 +ve and Bcl2 -ve tumours were significantly different (see Table 5.17).

Table 5.17 Survival times of Bcl2 +ve and Bcl2 -ve tumours.

	n	Min	Max	Mean	S.D	Median
Bcl2 +ve	37	2	134	67	54.1	65
Bcl2 -ve	68	4	138	32.1	41.5	16

The mean survival time in Bcl2 +ve tumours was little more than twice that of Bcl2 -ve tumours. On Mann Whitney U test this was statistically significant, $p = 0.013$.

Discussion

Over-expression of Bcl2 is expected to reduce the apoptotic rate of malignant cells and thus aid in tumour expansion. Therefore, theoretically, one would expect Bcl2 positive tumours to be associated with a shorter survival. However, the results above indicate a correlation in the opposite direction. This was not an isolated finding and several other investigators of lung cancer have reported similar results (e.g. Pezzella *et al.*, 1993; Fontanini *et al.*, 1995). The fact that in our series there was a some *positive* correlation between Bcl2 and apoptosis probably does not entirely explain the large difference in survival times that was noted.

In our subsequent analyses we sought other explanations for Bcl2's ability to prolong survival.

5.4.6 Bcl2, disease stage and lymph node metastasis

We investigated if there was any difference in Bcl2 expression among patients in different disease stages and if the Bcl2 index influenced in any way the propensity of a tumour to spread to regional lymph nodes. The Bcl2 indices of patients in the different disease stages can be seen in Table 5.18.

Table 5.18 Bcl2 indices of tumours in different disease stages.

	n	Min	Max	Mean	S.D.	Median
Stage I	45	0	100	37.4	42.6	16.2
Stage II	47	0	100	25.9	37.3	4.8
Stage IIIa	27	0	100	29.1	39.8	4.2
Stage IIIb	11	0	100	17.2	34	0
Stage IV	4	0	100	40.3	47.8	30.6

There was no visible trend in the mean Bcl2 indices in successively higher disease stages.

On Mann Whitney U test, no two means differed significantly.

The Bcl2 indices of patients in all three groups of lymph node involvement i.e. N0, N1 and N2 are described in Table 5.19.

Table 5.19 Bcl2 indices of tumours grouped according to lymph node status.

	n	Min	Max	Mean	S.D.	Median
N0	54	0	100	32.6	41.5	1.7
N1	62	0	100	27.4	37.9	4.2
N2	16	0	100	29.7	40.1	8.7

On Mann Whitney U test, there was no significant difference between the means.

Conclusion

Bcl2 expression in lung cancer does not influence disease stage or the ability to spread to regional lymph nodes.

5.4.7 Bcl2 and other pathological markers

To check if the influence of Bcl2 on survival was the result of its effect on anything other than apoptosis we investigated for possible correlation between Bcl2 expression and other pathological markers. The other markers that we checked were differentiation, AgNOR and ploidy. The general pathological significance of these markers has already been discussed in previous chapters (See sections 5.3.4, 5.3.5 and 5.3.6 respectively for discussions on these topics).

Bcl2 and differentiation

Bcl2 indices in each of the three groups—well differentiated, moderately differentiated and poorly differentiated tumours are described in Table 5.20.

Table 5.20 Bcl2 indices of tumours in different grades of histological differentiation.

	n	Min	Max	Mean	S.D	Median
Well diff.	12	0	100	24	43.1	0
Mod. diff.	58	0	100	30.4	40.5	3.3
Poorly diff	44	0	100	34.4	40.6	11.1

Although there was a discernible trend for the mean Bcl2 index to increase with worsening histological differentiation, these differences were not significant on Mann Whitney U test.

Bcl2 and AgNOR

Spearman rank correlation test was performed between B.I and AgNOR counts. No correlation was found between the two, $R_s = 0.0108$ and $p = 0.764$.

Bcl2 and ploidy

Bcl2 indices of tumours that were diploid or aneuploid can be seen in Table 5.21.

Table 5.21 Bcl2 indices of diploid and aneuploid tumours.

	n	Min	Max	Mean	S.D.	Median
Diploid	44	0	100	22.3	35	1.2
Aneuploid	63	0	100	35.4	42.6	8

Aneuploid tumours had a higher mean Bcl2 index but this was not statistically significant, $p = 0.160$.

Conclusion

Thus a higher Bcl2 index was seen in aneuploid tumours and in histologically less well differentiated tumours (both statistically insignificant trends). Both these are, in general, considered negative prognostic factors and therefore it still does not explain why a high Bcl2 index should be associated with a better prognosis. One must remember that in this particular series of lung tumours neither ploidy nor histological differentiation could be demonstrated to have any independent prognostic significance.

5.4.8 CD40 and apoptosis

In lymphocytes when CD40 is bound by its ligand, apoptosis is inhibited (see Section 1.4.4.1). To investigate if the expression of CD40 in lung cancer cells had a similar effect on apoptosis we performed Spearman rank correlation test between CD40 Index (C.I.) and A.I. No correlation was seen between the two, $R_s = 0.1088$ and $p = 0.211$. This lack of correlation is also very evident from the graph shown in Fig. 5.18.

We repeated the analysis after excluding the 78 cases which had a C.I. of zero. Again no correlation could be demonstrated, $R_s = 0.0223$ and $p = 0.870$. See Fig. 5.19.

We then looked for differences in A.I. between CD40 positive and CD40 negative tumours. See Table 5.22

Table 5.22 Apoptotic indices of CD40 +ve and CD40 -ve tumours.

	n	Min	Max	Mean	S.D.	Median
CD40 +ve	29	3.6	108.7	33.1	29.5	20.8
CD40 -ve	105	2.6	145.7	29.3	23.3	22.4

On Mann Whitney U test there was no significant difference between the means, $p=0.926$.

Finally we looked at High A.I. and Low A.I. tumours and investigated if there was any substantial difference in CD40 expression. See Table 5.23. The table below describes the CD40 indices of High AI and Low AI tumours.

Table 5.23 CD40 indices of high AI and low AI tumours.

	n	Min	Max	Mean	S.D.	Median
High AI	33	0	100	27.3	41.6	1.2
Low AI	101	0	100	20.5	34.8	0

On Mann Whitney U test there was no significant difference between the means, $p=0.280$.

Conclusion

Thus CD40 expression in lung cancer does not seem to influence the rate of apoptosis.

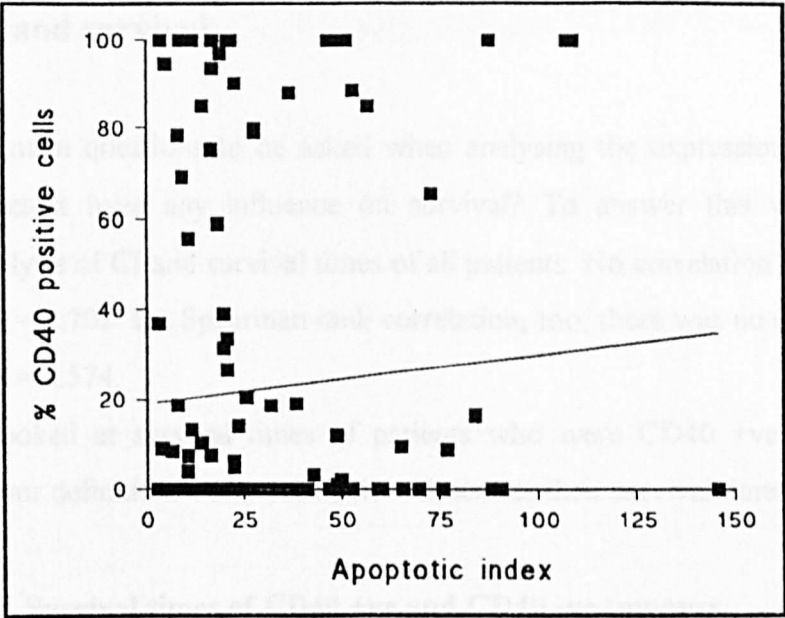


Fig. 5.18 Correlation of CD40 index and apoptotic index of lung tumours

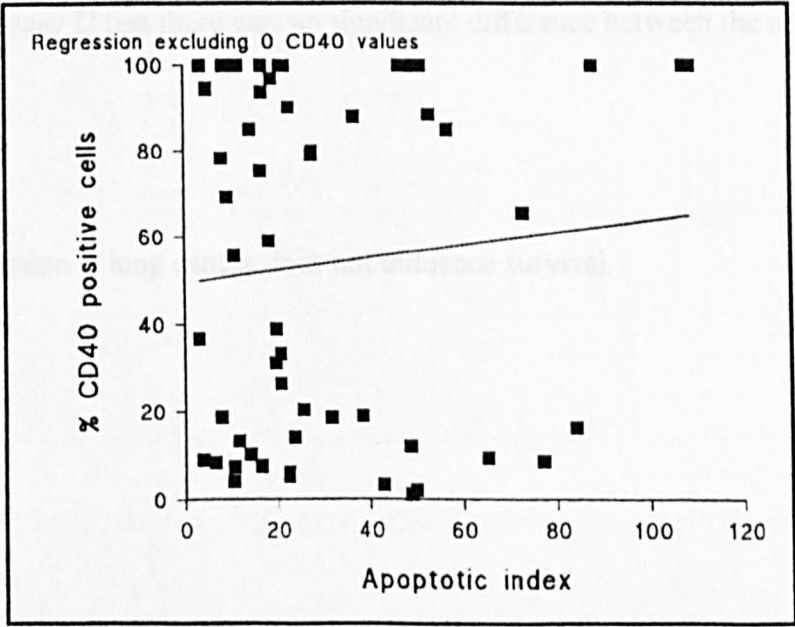


Fig. 5.19 Correlation of CD40 index and apoptotic index of lung tumours (excluding zero CD40 values).

5.4.9 CD40 and survival

One of the main questions to be asked when analysing the expression of a gene in a cancer is, does it have any influence on survival? To answer this we performed a regression analysis of CI and survival times of all patients. No correlation was evident, $r = -0.0376$ and $p = 0.702$. On Spearman rank correlation, too, there was no correlation, $R_s = -0.0553$ and $p = 0.574$.

Next we looked at survival times of patients who were CD40 +ve and CD40 -ve according to our definition. Table 5.24 below describes their survival times.

Table 5.24 Survival times of CD40 +ve and CD40 -ve tumours.

	n	Min	Max	Mean	S.D.	Median
CD40 +ve	25	3	133	40.7	48.2	15
CD40 -ve	80	2	138	44.1	48.8	18.5

On Mann Whitney U test there was no significant difference between the means, $p = 0.285$.

Conclusion

CD40 expression in lung cancer does not influence survival.

5.4.10 CD40, disease stage and lymph node metastasis

We investigated if there was any difference in CD40 expression in patients in different disease stages and if CD40 expression influenced the ability of a tumour to spread to regional lymph nodes. Table 5.25 below describes the CD40 indices of patients in different disease stages. There were four patients in Stage IV all of whom had a CD40 Index of zero.

Table 5.25 CD40 indices of tumours in different disease stages.

	n	Min	Max	Mean	S.D.	Median
Stage I	45	0	100	20.2	35.4	0
Stage II	47	0	100	26.2	38.5	0
Stage IIIa	27	0	100	20	34.8	0
Stage IIIb	11	0	100	26.1	44.4	0

On Mann Whitney U test there was no significant difference between the four means.

Next we tabulated the CD40 indices of patients in all three groups of lymph node involvement i.e. N0, N1 and N2 (Table 5.26).

Table 5.26 CD40 indices of tumours grouped according to lymph node status.

	n	Min	Max	Mean	S.D.	Median
N0	54	0	100	21.6	36.5	0
N1	62	0	100	25	38.3	0
N2	16	0	100	16.1	32.3	0

On Mann Whitney U test there was no significant difference between the means.

Conclusion

CD40 expression in lung cancer does not influence disease stage or the ability to spread to regional lymph nodes.

5.4.11 CD40 and other pathological markers

Next we investigated if there was any correlation between CD40 and other pathological markers e.g. differentiation, AgNOR and ploidy.

CD40 and differentiation

CD40 indices of patients with well, moderate and poorly differentiated tumours are described in Table 5.27.

Table 5.27 CD40 indices of tumours in different grades of histological differentiation.

	n	Min	Max	Mean	S.D.	Median
Well diff	12	0	100	26.9	44	0
Mod. Diff	58	0	100	24.4	37.1	0.6
Poorly diff	44	0	100	20.6	35.3	0

On Mann Whitney U test there was no significant difference between the means.

CD40 and AgNOR

Spearman rank correlation test was done between CI and AgNOR in all patients. No correlation could be demonstrated, $R_s = 0.0849$ and $p = 0.892$.

CD40 and ploidy

The CD40 indices of diploid and aneuploid tumours can be seen in Table 5.28 below.

Table 5.28 CD40 indices of diploid and aneuploid tumours.

	n	Min	Max	Mean	S.D.	Median
Diploid	44	0	100	32.3	40.9	8.7
Aneuploid	63	0	100	15.6	31.7	0

On Mann Whitney U test the mean CD40 indices were significantly different, $p=0.029$.

Conclusions

Thus, a higher CD40 index was associated with diploid tumours. There was no difference in CD40 expression among other pathological groups created on the basis of disease stage or histological differentiation.

5.4.12 Bcl2, CD40, apoptosis and survival

Having looked at Bcl2 and CD40 expression in isolation we next investigated if the two taken together had any influence on apoptosis or survival. Bcl2 and CD40 are both anti-apoptotic and there are suggestions that they might act synergistically (see Section 1.4.5.3). Thus one could expect their co-expression in tumours to have an additive effect on apoptosis or survival.

Tumours were divided into four groups according to their Bcl2 and CD40 status as defined in Sections 5.4.1 and 5.4.2.

a) Bcl2 -ve and CD40 -ve	BLCL
b) Bcl2 +ve and CD40 -ve	BHCL
c) Bcl2 -ve and CD40 +ve	BLCH
d) Bcl2 +ve and CD40 +ve	BHCH

The apoptotic indices in each of these four groups is described in Table 5.29.

Table 5.29 Apoptotic indices in four groups created according to Bcl2 and CD40 status.

	n	Min	Max	Mean	S.D.	Median
BLCL	69	3.1	145.7	26.9	24.7	19.8
BHCL	36	2.6	88.4	34	20	28.5
BLCH	23	3.6	107.2	29.2	24.9	20.8
BHCH	6	9.2	108.7	47.8	42.6	34.7

Only one pair of means were statistically different. When BLCL was compared to BHCL by Mann Whitney U test, $p = 0.010$. There was no significant difference between the other means.

The survival times in each of the four groups is described in Table 5.30.

Table 5.30 Survival times in four groups created according to Bcl2 and CD40 status.

	n	Min	Max	Mean	S.D.	Median
BLCL	49	5	138	29.7	38.8	16
BHCL	31	2	134	66.9	54.6	54
BLCH	19	4	133	38.1	48.3	15
BHCH	6	3	128	49.1	51.2	41

BHCL differed significantly from BLCL ($p = 0.014$) and there was tendency to significance when compared to BLCH ($p = 0.067$). The p values between the other pairs of means were not significant.

Within each of the four groups we performed Spearman rank correlation test between AI and survival. The coefficients of correlation are shown in Table 5.31.

Table 5.31 Coefficients of correlation between apoptosis and survival in four groups created according to Bcl2 and CD40 status.

	Rs	p
BLCL	0.0019	0.996
BHCL	-0.0944	0.614
BLCH	-0.0640	0.786
BHCH	-0.3714	>0.05

None of the p values were significant.

Conclusions

There was no correlation between apoptosis and survival in any of the groups. Thus even when differences of Bcl2 and CD40 expression were eliminated, apoptosis in lung cancer did not influence survival.

AI in the two Bcl2 positive groups was higher than that in the two corresponding Bcl2 negative groups. This might be a reflection of our previous finding (in Section 5.4.4) that AI was higher in Bcl2 +ve cases compared to Bcl2 -ve cases. One could conclude that Bcl2's effect on apoptotic rate in this series of cases is not neutralised by differences in CD40 expression. It must be remembered that CD40 expression on its own was found to have no effect on apoptotic rate (Section 5.4.8).

Among the four groups, those in BHCL survived the longest (66.9 months) and those in BLCL the shortest (29.7 months). This difference was significant statistically ($p = 0.014$). This is in parallel with the large difference in survival that was noted when Bcl2 expression was considered on its own (See section 5.4.5). In contrast there was little difference in survival between BHCH and BLCH ($p = 0.634$). Thus the expression of CD40 seems to have negated the survival prolonging effects of Bcl2.

We have already established that CD40 does not influence apoptotic rate either on its own or in conjunction with Bcl2. Could it be that CD40 influences some other biological action of Bcl2? Although the only *known* biological action of Bcl2 is prevention of

apoptosis, it is almost impossible to explain its salutary effect in NSCLC on the basis of this fact. Several authors have speculated that there must exist some other biological role for Bcl2 in lung cancer. To add fuel to this speculation, Bcl2 expression has been found to paradoxically inhibit the growth of some solid tumour cell lines (derived from colon, lung and brain) in culture (Pietenpol *et al.*, 1994). Is the expression of this speculated alternative role of Bcl2 only confined to specific tissues e.g. lung tissue? And is CD40 really involved in this role as our data would seem to indicate?

These questions will be dealt with in depth in the General Discussions Section.

5.5 Measurement of apoptosis in cultured tumour cells

The assessment of apoptosis in histological sections, no matter how specific and sensitive the staining technique employed and no matter how accurate the counting protocol used, is limited in its scope. Indices obtained by such methods might provide a true description of the state of apoptosis at the moment that the biopsy specimen was obtained, but they can say very little about the kinetics of the process. Apoptosis is after all a dynamic process and at any given moment of time the amount of apoptosis detectable within a tissue is a balance between the process of production of apoptotic bodies and the process of phagocytosis by neighbouring cells and macrophages. These processes vary independently and by all accounts they can both be very rapid and a high rate of production might be balanced by an equally high rate of phagocytosis resulting in very little detectable apoptosis (see Section 1.2.2). This would misrepresent the effect that apoptosis may have on cell numbers in a tumour. Histological sections are unable to provide any information about these nuances.

Theoretically, one way of studying the underlying dynamics of apoptosis in tumours would be to carry out serial measurements of apoptosis of tumour cells. For this the cells would need to be in culture from where they could be sampled at pre-set time intervals. From these serial measurements one could try to calculate a rate of apoptosis since there would be no phagocytosis. If this scheme could be implemented on disaggregated fresh tumours (rather than on cell lines) one could potentially obtain data about the state of affairs that exists in tumours *in vivo*. Such data would have considerable biological and clinical value. However, an important proviso is that the tumour cells would be removed from the influence of cytokines and growth factors *in vitro* and of course the tissue architecture would be destroyed, eliminating the influence of interactions between tumour cells and other cells and the extra cellular matrix (see Section 1.2.3.2).

We wanted to investigate if this was a feasible option for lung tumours. For this the tumours would first need to be disaggregated into a single cell suspension and cultured in medium. There are several ways by which to measure apoptosis in cell suspensions. Our

main method was to stain the cells with propidium iodide (PI) and determine the subdiploid fractions in DNA histograms. This method has been shown to be effective for leukaemia cells (Chant *et al.*, 1996). To distinguish tumour cells from non-tumour cells in the suspension we used the epithelial specific monoclonal antibody BerEP4 (see Section 3.1.5 a). Throughout the experiments we also used several other methods to measure apoptosis (cytospins, histological sections) to back up the results obtained by flow cytometry.

We chose to begin our experiments with malignant pleural effusions and then moved on to solid lung tumours.

5.5.1 Malignant pleural effusions

Introduction

There were several reasons to prefer to begin our experiments with pleural effusions rather than with solid lung tumours. Malignant pleural effusion is (sadly) a relatively common condition encountered in the medical wards (see Section 1.1.2.5). Aspiration of these effusions is a simple bedside procedure done under local anaesthetic. Within the effusion the malignant cells are held in a single cell suspension and thus there would be no need for complicated disaggregation protocols.

Apart from these practical considerations, there were some theoretical attractions as well. In bronchial epithelial cells (as indeed in many other cell types) apoptosis has been shown to be modulated by cell-extracellular matrix (ECM) and cell-cell interactions (see Section 1.2.3.2). Anchorage of cells to ECM sends a powerful survival message

mediated by integrins. Loss of contact with neighbouring cells and ECM makes a cell more prone to apoptosis (Aoshiba *et al.*, 1997). Thus it would be interesting to examine if malignant cells floating freely in the pleural cavity had a particularly high rate of apoptosis.

We collected malignant effusions from 10 patients whose details are tabulated below in Table 5.32. As described in the Methods Section (Section 4.2) we conducted a series of experiments on these effusions in an effort to measure apoptotic rate.

Table 5.32 Clinical and pathological parameters of pleural effusions used for cell culture experiments.

Sample	Age	Sex	Diagnosis	Amount of fluid collected
PE1	81	F	Bronchial carcinoma	1l
PE2	76	M	Bronchial carcinoma	1.5l
PE3	65	F	Bronchial carcinoma	1l
PE4	69	M	Bronchial carcinoma	1.2l
PE5	76	M	Adenocarcinoma—unknown site	1l
PE6	73	M	Bronchial carcinoma	1l
PE7	62	F	Metastatic breast cancer	1.4l
PE8	68	M	Adenocarcinoma—unknown site	1.5l
PE9	74	M	Mesothelioma	1l
PE10	72	M	Bronchial carcinoma	1.5l

Results

Unfortunately, our experiments on pleural effusions did not prove to be very informative. Only one effusion, PE8, produced the type of results that we had hoped to see. FACS analysis of a dual stained sample of this effusion clearly demonstrated two populations of cells - BerEP4 positive and negative (Fig. 5.20). A DNA histogram was obtained of the tumour cells (Fig. 5.21). This showed that 1.7% of the tumour cells were apoptotic on Day0. The corresponding figure for Days 1 and 2 were 7.6% and 13.2% respectively. (See Section 5.5.2.1 for a description of the analytic methods).

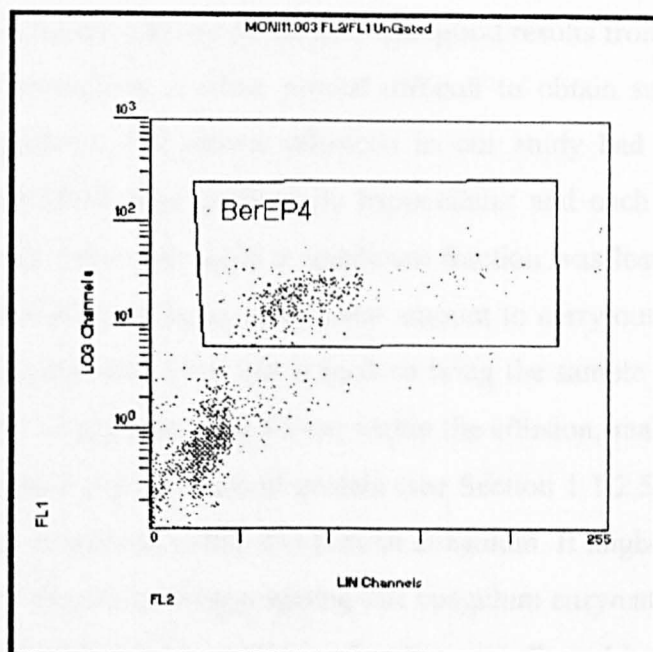


Fig. 5.20 Dot plot display of PE8 Day 0 sample. A gate has been drawn around the BerEP4 positive cells.

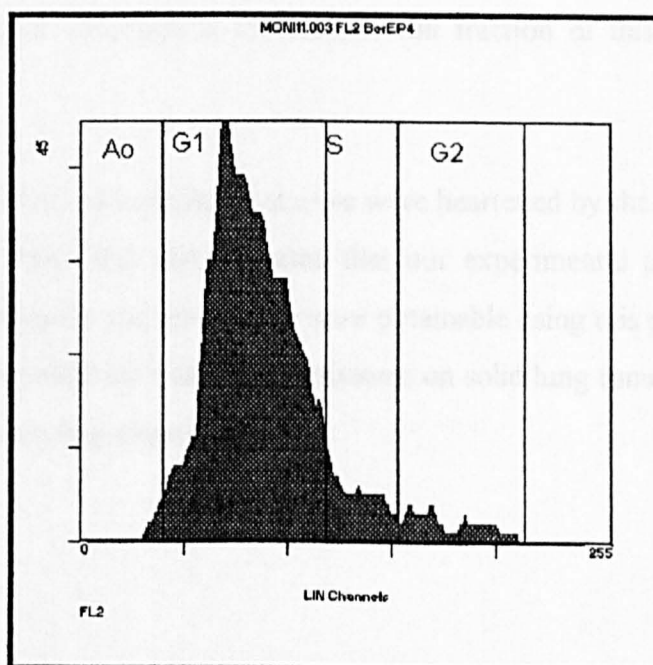


Fig. 5.21 DNA histogram of the BerEP4 positive gated cells in Fig. 5.20.

There were several reasons why we failed to obtain good results from the other samples. Contrary to our expectations, it often proved difficult to obtain sufficient numbers of tumour cells for analysis. The pleural effusions in our study had different grades of cellularity. PE4 and PE10 were particularly hypocellular and each yielded only about 500,000 cells in total. From this again a significant fraction was lost during procedures like washing and staining, leaving an insufficient amount to carry out all the experiments satisfactorily. During the short time that it took to bring the sample from the bedside to the laboratory, often a coagulum would form within the effusion, -malignant effusions are exudative and contain a large amount of protein (see Section 1.1.2.5). Some of the cells are likely to have been trapped within this protein coagulum. It might have been possible to increase the yield of cells by disaggregating this coagulum enzymatically.

The effusions contained variable numbers of malignant cells and in some samples these were largely outnumbered by non-malignant cells (i.e. lymphocytes, neutrophils and reactive mesothelial cells). PE3, PE6 and PE7 each contained less than 5% BerEP4 positive cells. There were also technical difficulties with staining. PE9 was a mesothelioma and therefore the tumour cells were BerEP4 negative. This prevented the proper assessment of apoptosis in the tumour cell fraction of this effusion. (see also Section 5.5.2.1).

In spite of these various disappointments, we were heartened by the positive results that we obtained with PE8. This demonstrated that our experimental protocol was indeed based on sound principles and that results *were* obtainable using this protocol.

We therefore proceeded with similar experiments on solid lung tumours. This proved to be a much more rewarding experience.

5.5.2 Fresh lung tumours

Twenty one freshly resected lung tumours were collected and analysed as per the protocol described in Section 4.2. The clinico-pathological details of these patients are described in Table 5.33 below.

Table 5.33 Clinical and histopathological parameters of fresh lung tumours used for cell culture experiments.

Sample	Age	Sex	Histology	Differentiation	Location	Size	Stage*
LC1	73	M	Squamous	Well	RLL	2.5cm	T1 N0 = I
LC2**	76	F	Adenocarcinoma	Well	RLL	2cm	T1 N0 = I
LC3	69	F	Squamous	Poor	LLL	6cm	T2 N1 = II
LC4	72	M	Squamous	Mod	RML	4cm	T2 N1 = II
LC5	85	M	Squamous	Poor	LLL	4cm	T2 N0 = I
LC6	54	M	Adenocarcinoma	Poor	LUL	4cm	T2 N1 = II
LC7	68	M	Squamous	Mod	LLL	4.5cm	T2 N1 = II
LC8	66	F	Squamous	Mod	RUL	3cm	T1 N0 = I
LC9	64	M	Squamous	Mod	LUL	7cm	T2 N1 = II
LC10	61	M	Adenocarcinoma	Poor	LLL	4.5cm	T2 N1 = II
LC11	73	F	Bronchoalveolar	Mod	RLL	5cm	T2 N0 = I
LC12	54	M	Squamous	Mod	LLL	6cm	T2 N1 = II
LC13	65	M	Adenocarcinoma	Mod	RLL	2cm	T1 N0 = I
LC14	53	M	Adenocarcinoma	Well	LUL	5cm	T2 N0 = I
LC15	62	F	Undifferentiated (NSCLC)	Undiff	LLL	4cm	T2 N0 = I
LC16	52	M	Actinomycosis	no	tumour	found	
LC17	63	F	Adenocarcinoma	Well	RUL	1.3cm	T1 N1 = II
LC18	74	M	Squamous	Poor	LLL	7cm	T2 N0 = I
LC19	76	F	Squamous	Poor	RML	4.5cm	T2 N1 = II
LC20	53	M	Leiomyosarcoma	Poor	RUL	11cm	T2 N0 = I
LC21	70	M	Squamous	Poor	LLL	4.5cm	T2 N1 = II

Key RUL = Right upper lobe

LUL = Left upper lobe

RML = Right middle lobe

LLL = Left lower lobe

RLL = Right lower lobe

* = Stage: No M value was assigned

**= Sample obtained from WGH, Coventry.

The main aim of these experiments was to obtain serial data on apoptotic rate using several methods in parallel e.g. flow cytometry, analysis of cytopins, analysis of histological sections stained with H&E and anti-ASP. Data from each of these

experiments have been described and summarised in the next few sections, while in the final section (5.5.2.6), apoptotic indices of Day0 of individual tumours have been tabulated for comparison. The expression of the apoptosis controlling genes Bcl2 and CD40 in this series of lung tumours was also quantified. These results have been summarised in Section 5.5.2.5 and described in detail in Section 5.5.2.6.

5.5.2.1 Flow Cytometric Analysis

Our main method of determining the apoptotic rate of these tumours was by serial flow cytometric analysis of tumour cell suspensions. As described in Section 4.2 three aliquots of tumour cells were obtained at intervals of 24 hours and dual stained with BerEP4-FITC and PI (Section 4.2.11).

Results

Flow cytometric data obtained for each tumour was analysed and manipulated using the Becton Dickinson Consort 30 programme and also the Dakomate programme (Dako). The aim of the analysis was to isolate the BerEP4 population (presumed tumour cells as discussed in Section 3.1.5 a) by electronic gating so that the PI staining could be analysed separately for this population, so giving the proportion of apoptotic tumour cells. This procedure will be described in detail by analysis of the data from tumour LC14.

The data was first displayed as a dot plot using FL2 and FL1 as the x- and y-axes respectively (see Fig. 5.22). From the plot two distinct populations of cells can be easily distinguished based on FL1 staining i.e BerEP4 positivity. The low FL1 cells represent the non-tumour population i.e. fibrocytes, lymphocytes and endothelial cells which are not of epithelial origin and so are BerEP4 negative. The high FL1 cells represent the tumour population. G1 and G2 can be identified in both the groups.

To analyse the tumour cells separately, a gate was drawn around the high FL1 cells (Fig. 5.23). Events at the extreme left of the plot were excluded as they were likely to represent sub-cellular debris. Similarly, events at the extreme right were likely to be multicellular clumps and were therefore avoided.

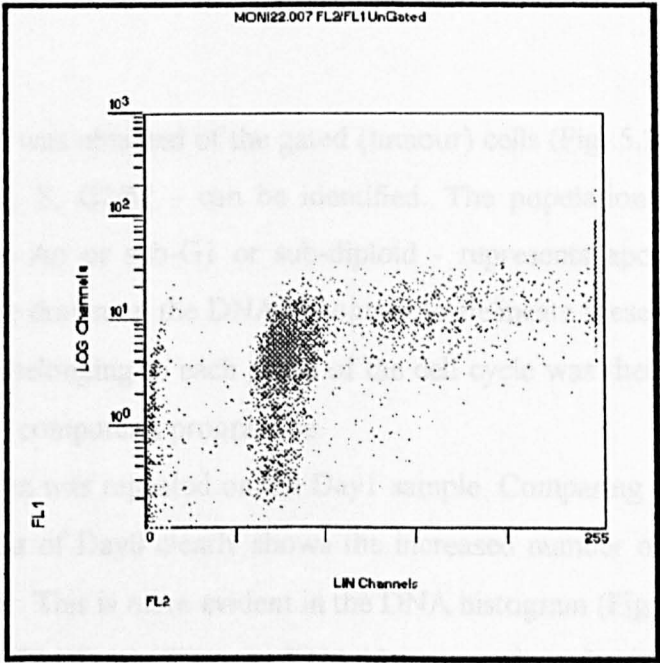


Fig.5.22 Dot plot display of LC14 Day0 showing two populations of cells.

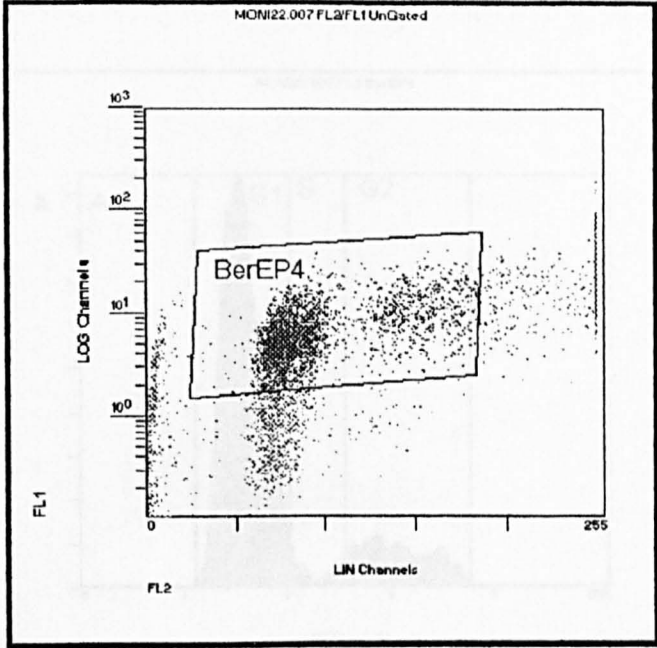


Fig. 5.23 Dot plot display of LC14 Day0 with a gate drawn around the BerEP4 positive cells.

A DNA histogram was obtained of the gated (tumour) cells (Fig. 5.24). All the phases of the cell cycle - G1, S, G2/M - can be identified. The population to the left of G1 - variously known as Ao or sub-G1 or sub-diploid - represents apoptotic cells (Section 3.1.5 a). Gates were drawn on the DNA histogram to delineate these subpopulations. The percentage of cells belonging to each phase of the cell cycle was then calculated from the histogram using the computer's programme.

The same operation was repeated on the Day1 sample. Comparing the dot plot of Day1 (Fig. 5.25) with that of Day0 clearly shows the increased number of cells in the sub-G1 region of the former. This is more evident in the DNA histogram (Fig. 5.26) obtained from the gated cells of LC14 Day1. The two DNA histograms have been overlaid in Fig. 5.27 to show the stark contrast in the Ao region.

Fig. 5.25 Dot plot of LC14 Day1 with a gate drawn around the BerEP4 positive cells.

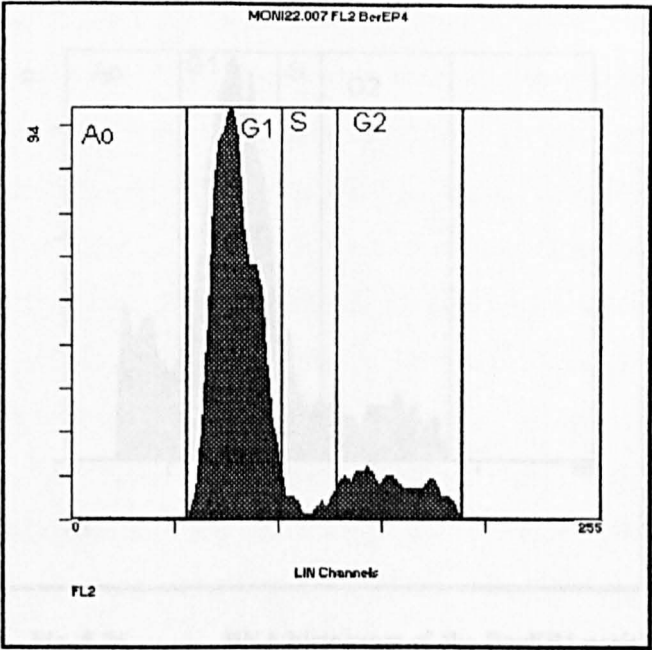


Fig. 5.24 DNA histogram of the BerEP4 positive cells in LC14 Day0.

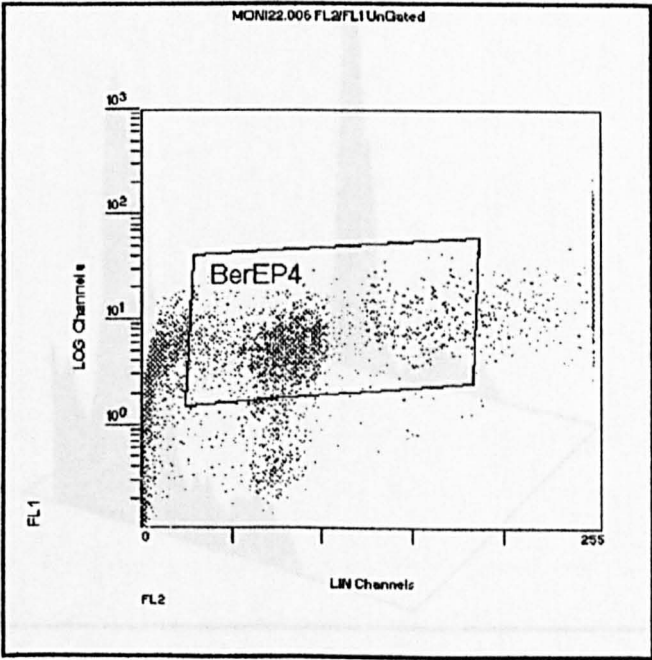


Fig. 5.25 Dot plot of LC14 Day1 with a gate drawn around the BerEP4 positive cells.

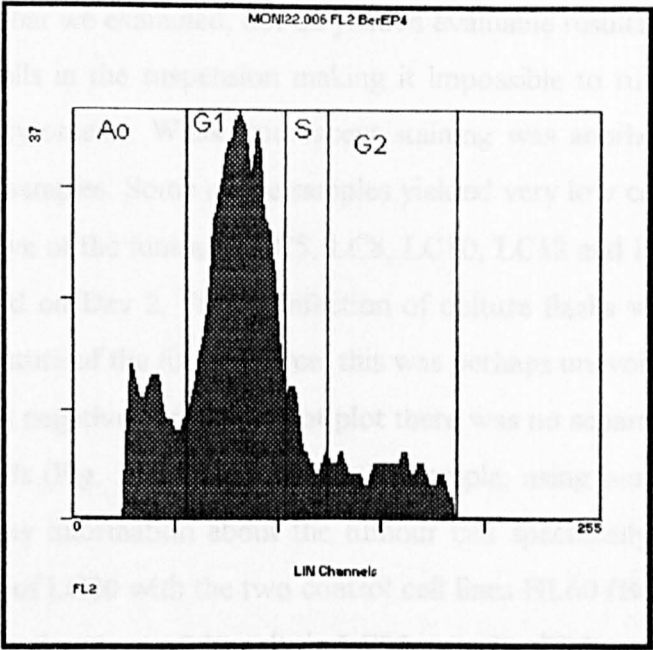


Fig. 5.26 DNA histogram of the BerEP4 positive cells of LC14 Day1.

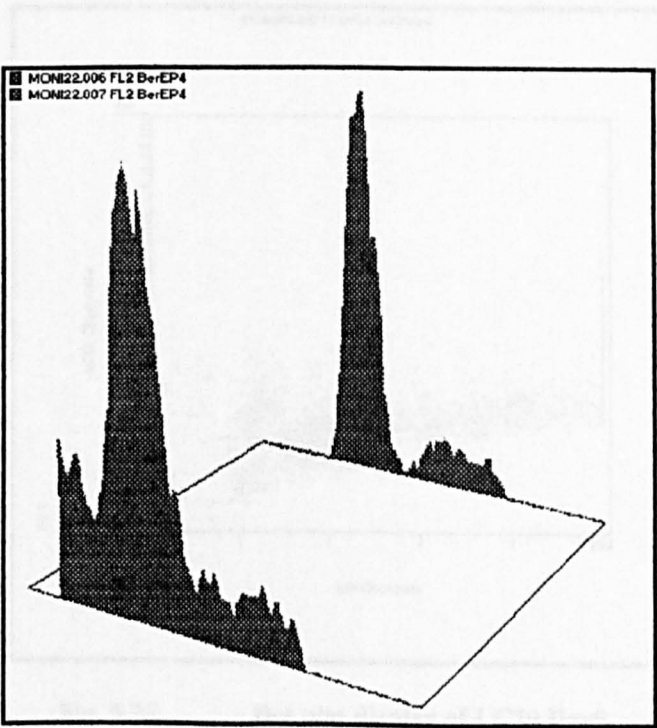


Fig. 5.27 Overlay of the DNA histograms obtained from LC14 on two consecutive days showing an increase in apoptosis with time.

Of the 21 tumours that we examined, not all yielded evaluable results. A common problem was clumping of cells in the suspension making it impossible to run the sample without clogging the flow cytometer. Weak fluorescent staining was another problem especially with the initial few samples. Some of the samples yielded very low cell numbers especially at 48 hours. Only five of the tumours (LC5, LC8, LC10, LC12 and LC21) yielded enough cells to be evaluated on Day 2. Fungal infection of culture flasks was another cause for failure. Given the nature of the tissue source, this was perhaps unavoidable.

LC20 was BerEP4 negative and on the dot plot there was no separation between tumour and non-tumour cells (Fig. 5.28). Thus, from this sample, using our protocol, it was not possible to glean any information about the tumour cell specifically. Comparison of the BerEP4 histograms of LC20 with the two control cell lines HL60 (BerEP4 -ve) and OE33 (BerEP4 +ve) shows that most of the cells in LC20 were BerEP4 negative (Fig. 5.29).

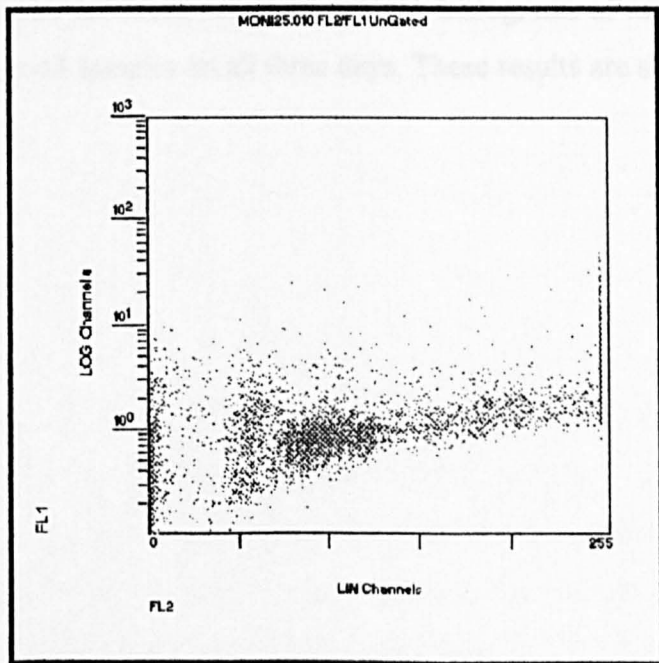


Fig. 5.28

Dot plot display of LC20 Day0.

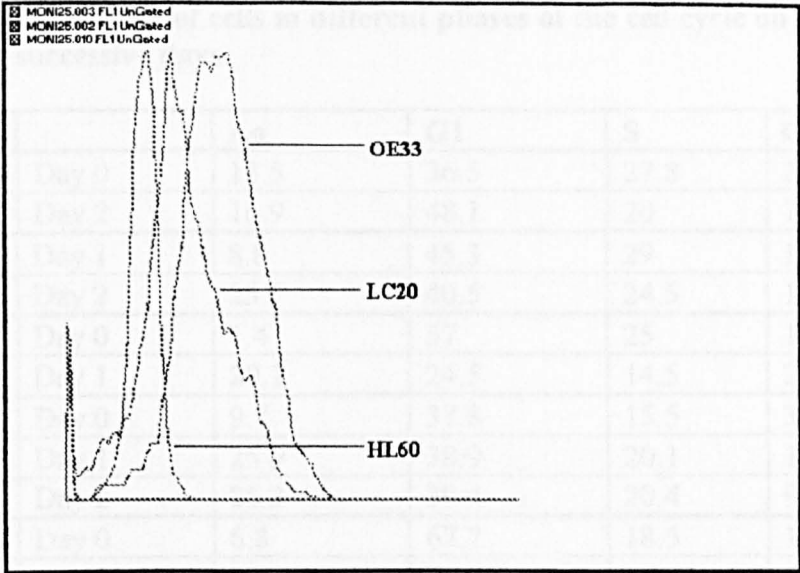


Fig. 5.29 Comparison of BerEP4 histograms of LC20 with OE33 and HL60 cell lines.

Altogether 11 samples produced informative DNA histograms of tumour cells. Of these only two produced good samples on all three days. These results are shown in Table 5.34.

It can be seen that the fraction of cells in the different phases of the cell cycle differed widely from cell tumour to another. As an example the values of Day0 are described in Table 5.33. The wide range for each phase emphasizes the wide differences in cell kinetics that exist even among tumours. Such diversity is due to the presence of multiple variables which control the passage of a cell through the cell cycle.

Table 5.34 Percentage of cells in different phases of the cell cycle on successive days.

		Ao	G1	S	G2
LC 5	Day 0	13.5	36.5	27.8	23.2
	Day 2	16.9	48.1	20	13.5
LC 8	Day 1	8.8	45.3	29	13.7
	Day 2	25.7	40.5	24.5	11
LC 9	Day 0	1.4	57	25	10
	Day 1	29.1	24.5	14.5	20.3
LC 10	Day 0	9.7	37.8	15.5	30.2
	Day 1	25.9	38.9	20.1	14.5
	Day 2	26.3	39.4	20.4	9.1
LC 11	Day 0	6.8	67.7	18.5	11.3
	Day 1	9.7	64.7	16.6	12.1
LC 12	Day 0	14.8	64.7	12.8	6.4
	Day 2	28.5	51	9.1	6.1
LC 14	Day 0	0.7	73.8	9.5	11.4
	Day 1	20.9	54.5	14.9	9
LC 15	Day 0	14.2	66.6	11.6	6.1
	Day 1	16.3	49.9	16.7	13.2
LC 18	Day 0	5.5	48	17	19.5
	Day1	7.5	56.1	12	15.8
LC 19	Day 0	6	61.1	15	11.4
	Day 1	12.3	53.4	15.9	11.5
LC 21	Day 0	3.3	66	10.6	15.2
	Day 1	15.2	65.1	10.1	13.9
	Day 2	16.3	57.1	11.6	15.2

It can be seen that the fraction of cells in the different phases of the cell cycle differed widely from one tumour to another. As an example the values of Day0 are described in Table 5.35. The wide range for each phase emphasises the wide differences in cell kinetics that must exist among tumours. Such diversity is due to the presence of multiple variables which control the passage of a cell through the cell cycle.

Table 5.35 Summary of Day0 cell cycle fractions in the fresh lung tumours shown in Table 5.34.

	Min	Max	Mean	SD	Median
Ao	0.7	14.8	7.7	4.9	6.8
G1	36.5	73.8	56.7	12.8	61.1
S	9.5	29	17.4	6.8	15.5
G2	6.1	30.2	14.4	7.3	11.4

A close look at Table 5.34 will show that while the fraction of cells in the other phases usually declined with time, the fraction of apoptotic cells increased in every tumour that we examined. Since the measurements were carried out after a fixed interval of time (24 hours) it was possible from these values to calculate the rate of apoptosis (see Table 5.36). For instance, $[Ao(\text{Day1}) - Ao(\text{Day0})]/24$ hours would give the percentage increase of apoptotic cells per hour in the first 24 hours (AR1 in Table 5.36). Similarly, subtracting $Ao(\text{Day1})$ from $Ao(\text{Day2})$ (where this value was available) would provide the rate for the next 24 hours (AR2). In those instances where only Day0 and Day2 values were available (LC5 and LC12), the rate was calculated as $[Ao(\text{Day2}) - Ao(\text{Day0})]/48$ hours (AR3). It can be seen that apoptosis proceeds at different rates in different tumours. Also, in the same tumour the rate varies with time. In those two instances (LC10 and LC21) where it was possible to make such measurements, apoptotic rate was seen to drop sharply in the second 24 hours compared to the first 24 hours.

Another observation that can be made at this point is the apparent lack of correlation between the apoptotic rate and the fraction of apoptotic cells on Day0. For instance LC9, LC14 and LC21 all start off with small fractions of apoptotic cells on Day0 which give no indication of the sharp rise that is seen on the following day. Others (LC11, LC15 and LC18) contained a relatively larger fraction of apoptotic cells initially but subsequently failed to register a significant rise. These observations were statistically corroborated by a total lack of correlation between apoptotic rate and apoptotic fraction on Day0. On Spearman rank correlation, $R_s = -0.4182$ and $p = 0.201$.

Similarly, there was no correlation between apoptotic rate and apoptotic index obtained from the corresponding histological sections (AI). On Spearman rank correlation $R_s = -0.3727$ and $p = 0.259$. The significance of this finding is discussed later in Section 6.10.

Finally we looked for correlation between apoptosis and proliferation as we had done with the archival lung tumours (Section 5.3.5). Instead of using AgNOR counts, here we used the S phase fraction as a measure of cell proliferation. No correlation was found on Spearman rank test between apoptotic rate and the S phase fraction of these tumours; $R_s = -0.0909$ and $p = 0.79$.

Table 5.36 Correlation of apoptotic rates with apoptotic fraction on Day0, AI from histological sections, and S phase fraction.

	AR1	AR2	AR3	Ao	AI	S
LC5			0.0708	13.5	65.3	27.8
LC8		0.7041		8.8	19.1	29
LC9	1.1541			1.4	26.7	25
LC10	0.675	0.0166		9.7	39.7	15.5
LC11	0.1208			6.8	18.1	18.5
LC12			0.5708	14.8	66.2	12.8
LC14	0.8416			0.7	20.1	9.5
LC15	0.0875			14.2	27.3	11.6
LC18	0.0833			5.5	48.8	17
LC19	0.2625			6	74.7	15
LC21	0.4958	0.0458		3.3	64.7	10.6

Key AR1 = Apoptotic rate between 0-24 hours
 AR2 = Apoptotic rate between 24-48 hours
 AR3 = Apoptotic rate between 0-48 hours.

Ao = Apoptotic fraction on Day 0
 AI = Apoptotic index from sections
 S = S phase fraction on Day 0.

5.5.2.2 Analysis of Cytospins

In parallel with flow cytometric analysis, we also attempted to measure apoptotic rate in the cultured tumour cells by serial examination of cytopins stained with H&E. For each tumour three cytospin slides were prepared at intervals of 24 hours. These slides were stained with haematoxylin and eosin and 10 fields were examined using an oil immersion lens ($\times 1000$). Apoptotic malignant cells were identified by the morphological criteria mentioned in Section 3.1.1. An apoptotic index (apoptotic cells/ 10,000 malignant cells) was calculated for each slide. These values have been tabulated below (Table 5.37). Not all tumours yielded cytospin slides of adequate quality for evaluation. In some tumours (LC6, LC7 and LC13) slides obtained on all three days only contained clumps of cells. In other instances cells were sparse and too few in number to yield any reliable results.

A quick comparison of the cytospin apoptotic indices (apoptosis/10,000 malignant cells) in Table 5.37 and the flow cytometric apoptotic fractions (apoptosis/100 malignant cells) in Table 5.36 will reveal that in the same sample, a larger number of cells were identified as apoptotic by FACS analysis than by morphological assessment. This indicates that cells might become subdiploid in DNA content before they acquire all the morphological features of apoptosis. However in comparison to the histological sections, there was a larger fraction of apoptotic cells in the cytospin preparations (see Table 5.41 in Section 5.5.2.6). This is not unexpected because in the culture flasks, cells are deprived of many of the anti-apoptotic signals that they would receive *in vivo* from close contact with other cells or with ECM (see Section 1.2.3.2).

In most lung tumours there was usually a rise in the fraction of apoptotic cells with time, but this was not invariable. In LC10, LC12 and LC15 the apoptotic index fell from Day0 to Day1 and in LC19 it fell from Day1 to Day2. Also, the rate of change of the apoptotic rate was not uniform but varied from the first 24 hours to the second 24 hours as in LC5, LC8 and LC9.

Table 5.37 Apoptotic indices obtained from H&E stained cytopspins on successive days.

Sample No.	Day 0	Day 1	Day 2
LC1	69	--	--
LC2	96	--	113
LC3	141	208	--
LC4	53	85	--
LC5	156	182	274
LC6	--	--	--
LC7	--	--	--
LC8	92	190	253
LC9	105	203	238
LC10	269	193	235
LC11	66	153	--
LC12	277	131	206
LC13	--	--	--
LC14	28	122	--
LC15	148	137	144
LC17	32	114	202
LC18	75	161	--
LC19	81	210	196
LC20	86	180	--
LC21	27	215	--

5.5.2.3 BerEP4 Staining

The BerEP4 status of all tumours was determined routinely by immunostaining of cytopspin preparations. Cells that express BerEP4 display a strong brown stain on the surface as well as in the cytoplasm (See Fig 5.30). Since this antigen is epithelial cell specific, positive staining can be used to distinguish between epithelial and non-epithelial cells. As discussed in the previous section, during FACS analysis, we used BerEP4-FITC staining as the sole criterion to distinguish tumour cells (of epithelial origin) from non-tumour cells i.e. fibrocytes, lymphocytes and endothelial cells. However, not all lung cancer cells are known to stain positively with this antibody (Latza *et al.*, 1990). Therefore, it was crucial to know the BerEP4 status of a tumour from the cytopspin to be able to properly interpret the results of FACS analysis.

Cytospins obtained on Day0 were immunostained with BerEP4 as described in Section 4.2.9. The stained slides were then examined under the oil immersion lens. The results are shown in Table 5.41.

The cytopins obtained from tumours LC6, LC7 and LC13 contained mainly clumps and it was impossible to determine the BerEP4 status of individual tumour cells. Tumour cells of LC20 were BerEP4 negative. The histological diagnosis of this tumour was leiomyosarcoma—a tumour arising from smooth muscle cells which do not express the BerEP4 antigen. As explained in section 5.6.2.1 this precluded the proper flow cytometric analysis of this tumour. All other tumours showed positive BerEP4 stained tumour cells.

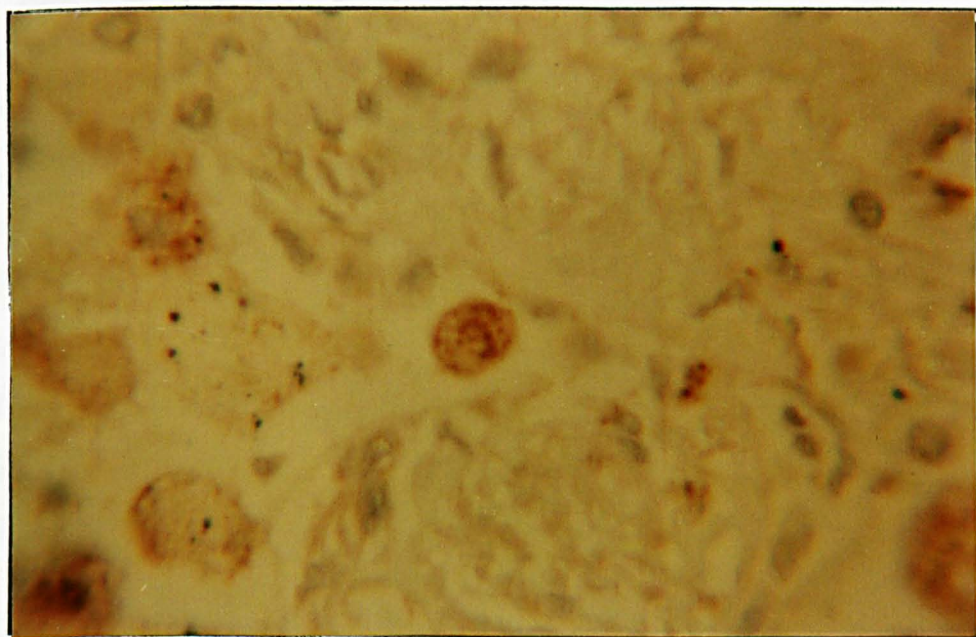


Fig. 5.30 Cytospin prepared from disaggregated lung tumour ($\times 630$) stained with monoclonal BerEP4 antibody. The large tumour cells show positive staining on the cell surface as well as within the cytoplasm.

5.5.2.4 Assessment of apoptosis in histological sections

To corroborate with the results obtained from flow cytometric analysis and cytopins, apoptosis was also assessed in the corresponding histological sections of

these tumours using H&E and ASP stains. 5µm paraffin sections were stained with H&E and ASP as described in Sections 4.2.1 and 4.2.4 respectively.

Haematoxylin and Eosin

H&E stained slides were examined under the oil immersion lens (×1,000) to identify apoptotic bodies by their morphological criteria i.e. condensed cytoplasm and pyknotic fragmented nuclei (See Section 3.1.1). Using the counting protocol described in Section 5.1 an apoptotic index (apoptotic cells/10,000 malignant cells) was calculated for each tumour. The indices for individual tumours are tabulated together with other data for comparison in Table 5.41. In Table 5.38 a summary of both AI(H&E) and AI(ASP) is given.

ASP

Sections stained with ASP were examined microscopically using the oil immersion lens (×1,000). As described in Section 3.1.4.2, with this staining method, other cells remain unstained but apoptotic cells are coloured brown thus aiding their identification. The counting protocol described in Section 5.2 was used to calculate an apoptotic index for each tumour. These indices [AI(ASP)] are shown in Table 5.41 and in Table 5.38 below.

Table 5.38 Apoptotic indices of fresh lung tumours assessed by H&E and ASP staining of histological sections.

	n	Min	Max	Mean	S.D.	Median
AI (H&E)	20	4.0	90.2	43.9	24.3	46.7
AI (ASP)	20	6.6	130.7	72.9	38.3	76.5

5.5.2.5 Assessment of Bcl2 and CD40 in histological sections

As an adjunct to the measurement of apoptotic rate, expression of two apoptosis controlling genes (Bcl2 and CD40) was also examined in the histological sections.

5µm paraffin sections were stained with Bcl2 and CD40 as described in protocols 4.2.2 and 4.2.3 respectively.

Bcl2 expression

Bcl2 stained slides were examined microscopically and a Bcl2 index (Bcl2 positive malignant cells/100 malignant cells) was calculated for each slide using the protocol described in Section 5.5.1. These indices (BI) have been shown in Table 5.40 and are summarised in Table 5.38 below. For comparison Bcl2 indices of the 134 squamous tumours that we studied separately (“Archival series”) have also been shown in Table 5.38.

Table 5.39 Bcl2 indices of fresh and archival lung tumours.

	n	Min	Max	Mean	Median	S.D.
All tumours	20	0	100	29.0	11.7	37.5
Only SCC	11	0	100	39.4	12.1	44.8
Only AC	6	0	72	15.0	4.9	28.1
Archival series	134	0	100	30.3	4.2	39.8

Of the 11 squamous cell carcinomas in this group 4 (36.3%) were Bcl2 positive (i.e. BI>50). In comparison in the Archival series of squamous cell lung carcinomas 31.6% were Bcl2 +ve.

Of the 6 adenocarcinomas only 1 (16.6%) was Bcl2 positive. The mean BI among adenocarcinomas was less than the mean BI among squamous cell carcinomas, but this difference was not significant ($p = 0.25$).

CD40 expression

Slides stained with anti-CD40 antibody were examined using the standard protocol (See Section 5.5.2) to calculate a CD40 index –CI (CD40 +ve malignant cells/100 malignant cells) for each lung tumour. These indices have been tabulated in Table 5.41 and Table 5.40 below. Again the CD40 indices of the archival series have been included in Table 5.40. for comparison.

Table 5.40 CD40 indices of fresh and archival lung tumours.

	n	Min	Max	Mean	Median	S.D.
All tumours	20	0	100	27.5	5.0	40.2
Only SCC	11	0	100	20.4	0	38.5
Only AC	6	0	100	45.6	36.9	50.2
Archival series	134	0	100	22.2	0	36.5

Of the 11 squamous cell carcinomas in this group 2 (18.1%) were CD40 positive (i.e. CI>50). In comparison in the Archival series of squamous cell lung carcinomas 21.8% were CD40 +ve.

Of the 6 adenocarcinomas in this group 3 (50%) were CD40 positive. The mean CI among adenocarcinomas was higher than the mean CI among squamous cell carcinomas, but this difference was not statistically significant ($p = 0.264$).

5.5.2.6 Correlation between the various methods of measuring apoptosis in cultured tumour cells

As discussed in Section 3.1 every method of measuring apoptosis has its own advantages and disadvantages. We had an ideal opportunity to compare some of these methods because during the course of our experiments on lung tumours we had used several methods in parallel to measure apoptosis on the same tumour samples. To facilitate comparison, data obtained by the different methods has been summarised in Table 5.41. The indices obtained from the sections AI(H&E) and AI(ASP) (the unit for both is apoptotic cells/10,000 malignant cells) can be thought to represent the apoptotic state of the tumour *in vivo*. Once the tumour was disaggregated and the cells had spent some time in culture medium the apoptotic state is likely to have changed considerably because in the new environment the cells would be deprived of many of the usual survival signals. But the apoptotic index obtained immediately after disaggregation (Day0) is still likely to bear some resemblance to the apoptotic state *in vivo*. Therefore when comparing the histological sections with the cultured cells, we only considered the Day0 apoptotic values of the latter.

Results

Flow cytometry and AI(H&E)

We performed a regression analysis between the Day0 FACS indices, AI(FACS) and the corresponding AI(H&E) values. There was a tendency towards correlation between these indices ($p=0.1010$) which was also apparent from the graph Fig 5.31. One must recall that when apoptotic *rate* rather than apoptotic fraction on Day0 was compared with AI(H&E) there was no relation between the two (Section 5.5.2.1).

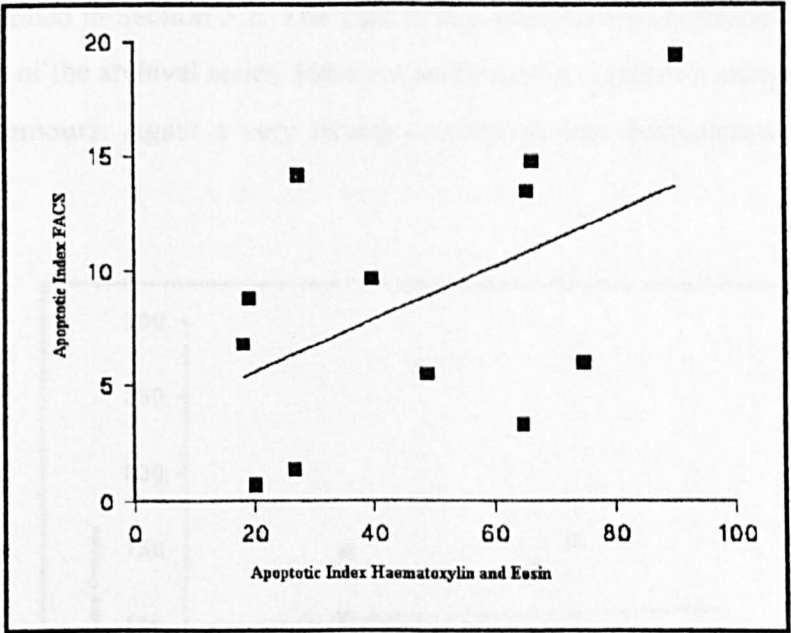


Fig. 5.31 Correlation between apoptotic index (FACS) and apoptotic index (H&E) of fresh lung tumours.

Cytospin and AI(H&E)

On regression analysis between AI(Cyto) and the corresponding AI(H&E) values, no correlation was apparent, $p = 0.8715$. See also Fig. 5.32.

Flow cytometry and cytopins

Next, a regression analysis was performed between the apoptotic indices obtained from flow cytometry and cytopins on Day0. A tendency towards correlation was noted. between these two sets of values ($p = 0.0818$). This is also evident in the graph (Fig. 5.33).

H&E and ASP

A strong correlation between these two methods of measuring apoptosis has already been established in Section 5.2. The data in that analysis also included 26 squamous cell carcinomas of the archival series. Here we performed a regression analysis exclusively on the fresh tumours. Again a very strong correlation was demonstrated, $p < 0.001$ and $r = 0.968$.

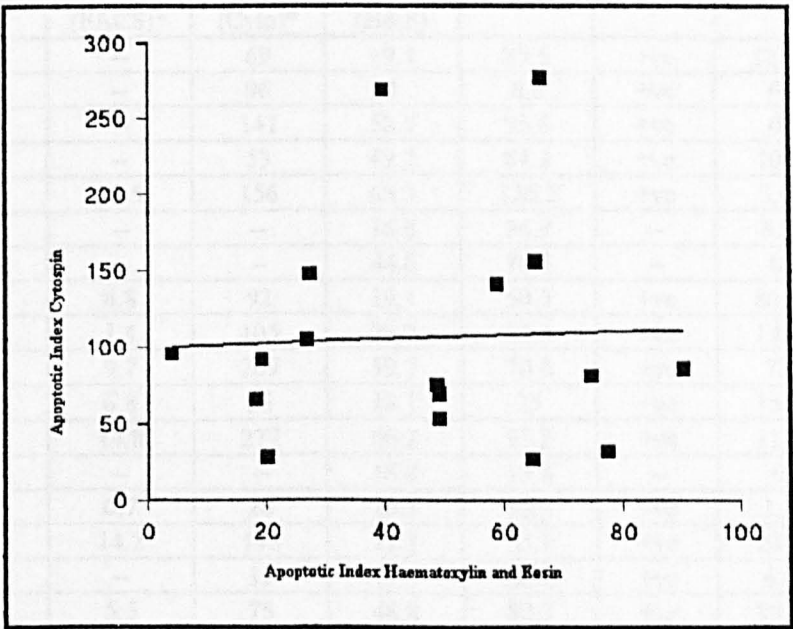


Fig. 5.32 Correlation between apoptotic index (cytospin) and apoptotic index (H&E) of fresh lung tumours.

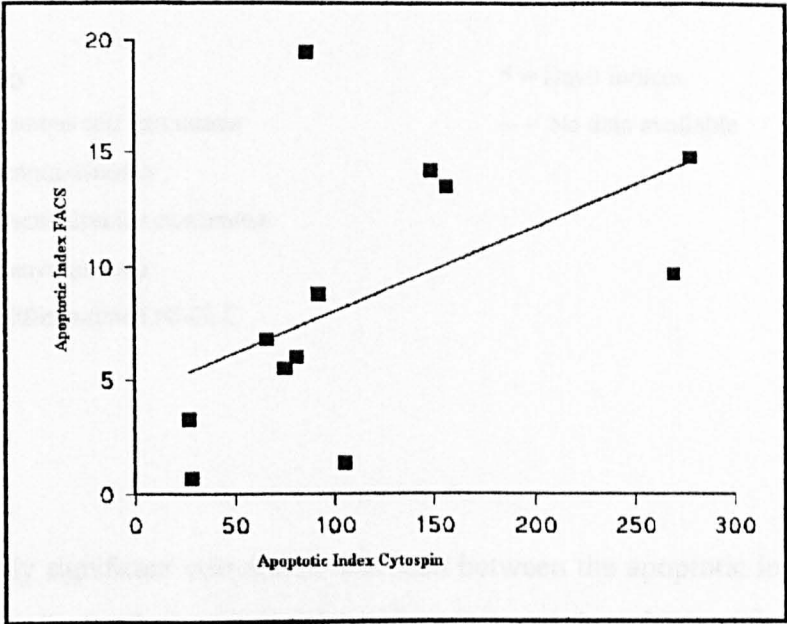


Fig. 5.33 Correlation between apoptotic index (FACS) and apoptotic index (cytospin) of the fresh lung tumours.

Table 5.41 Comparison of apoptotic indices of tumours measured by different methods and description of the expression of BerEP4, Bcl2 and CD40 in each tumour.

Serial No	Histo	AI (FACS)*	AI (Cyto)*	AI (H&E)	AI (ASP)	BerEP4	BI	CI
LC1	S	--	69	49.1	83.5	+ve	12.1	0
LC2	A	--	96	4	6.6	+ve	0	0
LC3	S	--	141	58.9	95.6	+ve	0	12.6
LC4	S	--	53	49.2	84.1	+ve	100	7.8
LC5	S	13.5	156	65.3	126.5	+ve	3.6	0
LC6	A	--	--	16.8	36.9	--	8.5	0
LC7	S	--	--	44.6	70.5	--	0	0
LC8	S	8.8	92	19.1	50.5	+ve	86.3	95.6
LC9	S	1.4	105	26.7	51.3	+ve	19.7	100
LC10	A	9.7	269	39.7	70.8	+ve	72	2.3
LC11	B	6.8	66	18.1	25	+ve	15.9	30.5
LC12	S	14.8	277	66.2	93.2	+ve	11.3	0
LC13	A	--	--	18.8	15.8	--	0	100
LC14	A	0.7	28	20.1	32.2	+ve	3.2	100
LC15	U	14.2	148	27.3	53.7	+ve	23.8	22.6
LC17	A	--	32	77.4	123.9	+ve	6.6	71.5
LC18	S	5.5	75	48.8	82.3	+ve	95.4	0
LC19	S	6	81	74.7	127	+ve	100	0
LC20	L	19.5	86	90.2	130.7	-ve	17.9	0
LC21	S	3.3	27	64.7	99.4	+ve	5.2	9

Key	Histology	* = Day0 indices
	S = Squamous cell carcinoma	-- = No data available
	A = Adenocarcinoma	
	B = Bronchoalveolar carcinoma	
	L = Leiomyosarcoma	
	U = Undifferentiated NSCLC	

Discussion

No statistically significant correlation was seen between the apoptotic indices obtained from cultured cells (by flow cytometry or cytopins) and that obtained from histological sections. This is not surprising because cells in the culture flask exist under conditions very different from that in the tumour nodules *in vivo*. Also several artefacts may distort the estimation of apoptosis in cultured cells. These issues are discussed more fully in Section 6.10.

CHAPTER 6

DISCUSSION

6.1 Introduction

Lung cancer is a dreadful disease and its incidence world wide is rising. We still do not possess adequate means of treating this disease. Development of new treatment protocols and new chemotherapeutic agents have not had a significant effect on the dismal mortality figures from this disease which have remained unchanged in the last 25 years. There are many reasons why lung cancer is so difficult to treat. When patients first present with symptoms often the disease is well advanced and therefore potentially curative treatment cannot be undertaken. For those with localised NSCLC surgical resection (lobectomy or pneumonectomy) offers the best chance of cure but it is not invariably successful. A significant fraction of those who are operated upon will die of local recurrence or distant metastasis within the first two years. To delay or prevent this from happening, adjuvant chemotherapy was introduced a few years ago. Chemotherapy is also often prescribed for those with advanced NSCLC who cannot be operated. Patients seem to vary in their response to chemotherapeutic treatment and even in those who do respond, the systemic side effects caused by such drugs often results in a very poor quality of life.

When, not long ago, the role of apoptosis in the biology of cancer was revealed, it kindled hope that a proper understanding of this process and its regulatory genes might provide answers to several of the problems faced by physicians dealing with lung cancer:

In animal models of carcinogenesis derangement of apoptosis is one of the earliest events in the neoplastic process (Schulte-Hermann *et al.*, 1981; Schulte-Hermann *et al.*, 1990; see also Section 1.2.5). It is expected that detection of such derangements in the pre-neoplastic lesions of high-risk individuals (i.e. heavy smokers) might hasten the process of diagnosis (see Section 1.1.4.3).

Secondly apoptosis might provide a good prognostic marker for lung cancer. It is rational to assume that a physiological process that leads to the elimination of cells should have at least some bearing on the outcome of lung cancer. Therefore quantifying

apoptosis within a tumour to arrive at some kind of apoptotic index should provide a valuable prognostic tool.

Thirdly, since it is now known that most forms of anti-cancer treatments produce their effects by inducing apoptosis in tumour cells (Lowe *et al.*, 1993; McDonnell *et al.*, 1992; see also Section 1.2.5.4), the ability of a tumour to undergo apoptosis, judged either by quantifying apoptotic cells or by assessing the state of the apoptosis controlling genes, should help to predict the tumours response to treatment.

Finally, it has been demonstrated that the process of apoptosis can be manipulated genetically. Both in cell culture experiments as well as in animal models cancer cells can be induced to die quickly by modifying their apoptosis controlling genes (Reed *et al.*, 1990; Debatin *et al.*, 1990). Such treatments have recently been tried successfully in small groups of patients with lung cancer (Roth *et al.*, 1996). Larger trials are needed before these modes of treatment become common practice.

But before such ambitious projects are undertaken some very fundamental questions still remain to be answered. In real life what is the relative importance of apoptosis in lung cancer when compared to other pathological parameters? Does the amount of apoptosis in a tumour help to predict outcome in an individual case? How do the genes regulating apoptosis affect clinical outcome? The answers to these questions are being sought in many laboratories throughout the world both by experiments on animal models and cell lines and also by analysis of histological material by standard staining techniques and immunological methods. The latter is particularly necessary because the situation *in vivo* in human cancer is often very different from that prevailing in cell cultures or animal models. Even if one were to ignore lymphoid neoplasms and consider only epithelial malignancies, wide differences will be seen to exist in the results obtained from various tumour types which is a reflection of the underlying biological diversity in cells of different lineage.

Even in tumours at the same site there are often conflicting reports. One of the reasons for such variation might be a lack of uniformity in gathering and reporting information. Several methods are available for measuring the same parameter and there are various

ways of reporting the same information which may make comparison impossible in some cases. For instance it is usual for apoptosis to be quantified in histological sections as apoptotic cells/100 malignant cells. However many investigators have reported it as apoptotic cells per high power field or apoptosis per mm². While this might serve the purpose of comparing the apoptotic rates of the various tumours in that particular series, it makes it virtually impossible to compare it to data obtained from elsewhere. Similarly, there is a singular lack of uniformity in reporting the results of immunohistochemical tests as different groups tend to use different cut-off points. What is reported as positive in one series could be regarded as negative by another group of investigators. What is worse is that often no indication is given of the criterion that was used to classify positive and negative. Therefore one needs to be cautious when interpreting results of clinical and laboratory investigations.

In spite of such reservations several conclusions can be confidently drawn from the data available. Some of these conclusions are the converse of what is known or can be deduced from known biological facts. This just highlights that great lacunae still must exist in our understanding of this most fundamental physiological process.

The aim of this thesis was to attempt to learn more about apoptosis in lung cancer, and relate this to the clinical situation. The experimental data obtained are presented and discussed in Chapter 5. The aim of this chapter is to put my results into the broader context of the study of apoptosis in cancer.

6.2 Quantification of apoptosis in cancer and pre-malignant conditions: the relationship of apoptosis to tumour progression.

To understand the role of apoptosis in lung cancer it is very important to first quantify it in cancer specimens so as to be able to relate it to other pathological markers and to outcome. In our series of 134 cases of squamous cell carcinoma (SCC), the apoptotic indices (examined by Haematoxylin and Eosin) ranged from 2.4/10,000 malignant cells to 145.5/10,000 malignant cells (see Section 5.1) i.e. 0.024% to 1.455% with a mean of 0.302% and SD 0.247% (conversion into percentage will facilitate comparison with other published results).

After an extensive search of literature we could find comparatively little quantitative data on the extent of apoptosis in lung cancer. A similar low frequency and wide range of apoptotic indices in lung cancer has been reported by other investigators. Stammers and Volm counted apoptosis using the TUNEL technique in a series of 178 cases of NSCLC (Stammers and Volm, 1996). They reported apoptotic indices that ranged from 0.0-1.7% (mean value \pm standard deviation: 0.37 ± 0.29 ; median 0.25%). These values are remarkably close to those that we obtained in our series.

Earlier Tormanen and co-workers had counted apoptosis in 75 cases of lung cancer [47 squamous cell cancers, 24 adenocarcinomas (AC), 3 small cell lung cancers (SCLC) and 1 large cell cancer] in order to look for prognostic significance (Tormanen *et al.*, 1995). They too had used the 3' end labelling technique to identify apoptosis. Among squamous cell carcinomas they found a mean apoptotic index of $0.38 \pm 0.05\%$ (mean \pm SD). In ACs they reported a higher index of $0.48 \pm 0.08\%$, while in their 3 SCLCs the index was even higher at $0.92 \pm 0.3\%$. In the 6 ACs in our series of 20 fresh lung tumours the AIs were not very different from that among the SCCs – 0.29 ± 0.26 (see Table 5.41). However the higher incidence of apoptosis in SCLCs has been confirmed by the only other group who have quantified apoptosis in this category of lung cancers. Eerola *et al*

have found a mean apoptotic index of 0.97 (range: 0.01-6.5%) in a series of 40 cases of SCLC (Eerola *et al.*, 1997).

Two other groups have counted apoptosis in lung cancers. Komaki *et al* found apoptotic indices to range from 0.2-2.8% (median 1.0%) in 173 cases of NSCLC (Komaki *et al.*, 1996). In their much smaller series of 38 lung cancers, Kargi *et al* found the median AI to be 9.52% (range: 2-26%) (Kargi *et al.*, 1997).

There are several quantitative studies of apoptosis in other epithelial tumours. In squamous cell carcinoma of the oropharynx and oral cavity, Birchall *et al* reported the mean AI to be $0.14 \pm 0.14\%$ and in SCC of the oesophagus Ohashi *et al* found AIs to range from 1.09-2.59% (Birchall *et al.*, 1995; Ohashi *et al.*, 1997). In carcinoma of the cervix (which is another example of squamous cell cancer) Sheets *et al* found a mean AI of $0.4 \pm 0.1\%$ (Sheets *et al.*, 1996).

In adenocarcinomas too the incidence of apoptosis is very low. In the prostate, apoptosis has been estimated as 1.7% (range: 0.2-2.4%) in nine solid undifferentiated carcinomas (Gaffney, 1994), between 0.09-1.73% in 26 adenocarcinomas (Colecchia *et al.*, 1997) and between 2.69-2.75% in 16 cases (Drachenburg *et al.*, 1997). In colorectal cancer Baretton *et al.* found a mean apoptotic index of $1.9 \pm 0.15\%$ in 93 cases (Baretton *et al.*, 1996).

None of the cases reported above had received any form of therapy which could have caused apoptosis (e.g. chemotherapy, radiotherapy, hormone ablation therapy) and therefore what was observed can be considered to be “spontaneous” apoptosis. It will be recalled that such spontaneous apoptosis in tumours is caused by both intrinsic factors like oncogenes (p53) that are activated by the presence of genetic abnormalities within the cell, and extrinsic factors like cytotoxic T cells and lack of nutrients and oxygen. It is generally presumed that as a lesion progresses from pre-malignancy to malignancy such spontaneous apoptosis decreases and as a consequence the tumour increases in size (see Section 1.2.5.3).

However this is not what is usually observed in real life. In most cases the incidence of apoptosis is seen to increase or remain static as a tumour progresses. In prostatic cancer both Drachenburg and Wheeler found apoptotic rates to rise from benign glands to high

grade intraepithelial neoplasia to adenocarcinoma (Drachenburg *et al.*, 1997; Wheeler *et al.*, 1994). In addition Tu *et al.* found a higher rate of apoptosis in foci of metastatic prostatic cancer than in localised cancer (Tu *et al.*, 1996). In squamous cell carcinoma of the oesophagus (Ohashi *et al.*, 1997) and oral cavity (Birchall *et al.*, 1995) apoptosis increased as lesions progressed from pre-malignancy to malignancy. Two groups of investigators have found the apoptotic indices in colonic carcinomas to be no different from that in the adenomas which precede them (Barreton *et al.*, 1996; Koike, 1996). However, another group (Bedi *et al.*, 1995) found a progressive inhibition of apoptosis during the transformation of normal colorectal epithelium to carcinoma. In cervical carcinoma Shoji *et al.* found a positive correlation between apoptosis and rising malignant grade i.e. cervical intraepithelial neoplasia, microinvasive carcinoma and invasive squamous cell carcinoma (Shoji *et al.*, 1996). Only Sheets *et al.* found a significant ($p < 0.001$) drop in apoptosis as the degree of cervical neoplasia increased (Sheets *et al.*, 1996).

In lung cancer we could find no such comparative study of apoptotic rates in pre-malignant and malignant lesions. Tormanen *et al.* had counted apoptosis in 11 samples of normal bronchial epithelium and found an apoptotic index of $0.02 \pm 0.02\%$ which was much lower than what they had found in any sample of malignant lung tissue (see above). Unlike cervical cancer and colonic cancer where it is standard clinical practice to obtain serial histological or cytological samples from patients known to be at risk of developing cancer, in the case of lung cancer obtaining such serial pre-neoplastic samples might prove to be very difficult. The only other alternative might be to take a sample of resected lung cancer and to examine the adjacent areas of normal and dysplastic epithelium for apoptosis. Such a study is urgently required to answer some very fundamental questions on the importance (or otherwise) of apoptosis during progression of lung cancer. The evidence available from other cancers would seem to indicate that decreased apoptotic cell loss is probably not an important factor during the stage of tumour progression, although in the earlier stages of neoplasia (i.e. initiation and promotion) apoptosis may play a more decisive role as indicated by the experiments on

rat hepatocarcinogenesis by Schulte-Hermann and co-workers (Schulte-Hermann *et al.*, 1981 and 1990).

Why does apoptosis seem to increase as the tumour progresses? Is increased apoptosis a marker of tumour progression or is it merely an epiphenomenon, a sign of rapid tumour growth outstripping the rate of new vessel formation? Theoretically such an imbalance would create more competition for nutrients and oxygen among the increasing number of new cells and inevitably lead to some amount of cell death by apoptosis. There is experimental evidence to support this contention. In transgenic mice treatment with the angiogenesis inhibitor AGM-1470 has been shown to result in a 40% reduction of new vessel density in islet cell tumours and a 100% increase in apoptotic rate (Parangi *et al.*, 1996). Other investigators have reported similar findings in separate models of animal carcinogenesis (Oreilly *et al.*, 1996; Saji *et al.*, 1997). Recently, Lu and Tanigawa have demonstrated that in gastric carcinoma, there is a highly significant inverse correlation between intratumoral microvessel densities and AI ($p < 0.0001$) (Lu and Tanigawa 1997). Theoretically it is at least conceivable that as the fledgling tumour increases in size, the rate of microvessel formation may not always be able to keep pace with the rate of cell proliferation. This imbalance would result in increased apoptosis.

Tumours seem to thrive inspite of this increasing death rate which indicates that the proliferative rate must usually keep up with and exceed the rate of cell loss. In other words the rate of cell loss is probably not an important determinant of growth once the tumour has proceeded beyond a certain phase of its natural history.

6.3 Apoptosis and survival

It might be expected that a high level of apoptosis suggesting slower tumour growth would be linked to better survival in lung cancer. In our series patients with a high apoptotic rate tended to have a shorter survival than the low apoptotic group, but this did not reach statistical significance ($p = 0.131$). No significant correlation between apoptosis and survival emerged even when various other pathological parameters like stage, grade proliferation rate and ploidy were corrected for (see Section 5.3).

Three other groups have investigated the value of apoptosis in predicting survival in lung cancer. Stammers and Volm divided the 178 NSCLC cases of their series into high apoptosis and low apoptosis according as they were above or below the median apoptotic index for the group. Those with a high apoptotic index had a mean survival time of 65 weeks while those with a low apoptotic index survived on average for 89 weeks. This difference was not statistically significant (Stammers and Volm, 1996).

Komaki *et al* undertook a retrospective analysis on 173 cases who had undergone lung resection for NSCLC. Their series included 86 SCCs, 73 adenocarcinomas (AC), 3 large cell carcinomas (LC), 6 adenosquamous carcinomas and 5 unclassified NSCLCs. Patients were observed from 2 to 209 months (median 27 months). The overall survival of the patients, which was 33% at 5 years, did not depend on the level of apoptosis. However, on multi-variate analysis, in the AC/LC group high apoptosis was associated with a significant chance of distant metastasis at 5 years (Komaki *et al.*, 1996).

Tormanen *et al* found a significant correlation between high apoptosis and shorter survival. Among their 75 lung cancers those with apoptotic indices greater than 1.5% showed a significantly shorter survival time than the patients with apoptotic indices of 1.5% or less ($p < 0.01$). By multivariate analysis enhanced apoptosis in NSCLC showed a 1.9 fold risk (95% CI, 1.04-3.60; $p = 0.04$) for a shortened survival (Tormanen *et al.*, 1995).

A similar trend between high apoptosis and short survival has also been noted in cancers at other sites. In carcinoma of the cervix (squamous cell carcinoma) Levine *et al*

found that patients with a high (greater than median) AI had a lower 5 year recurrence free rate ($p = 0.012$) as well as lower 5 year survival rate ($p = 0.003$) (Levine *et al.*, 1995). In a retrospective analysis of 400 cases of transitional cell bladder carcinoma, followed up for a mean of 9.5 years (range: 3.2-25.2 years), high AI was found to predict shorter survival ($p < 0.0001$) on univariate analysis but did not emerge as an independent prognostic factor on multivariate analysis (Lipponen and Aaltoma, 1994). Similarly in breast cancer, high AI predicted shorter survival in both axillary lymph node positive as well as lymph node negative groups, but was shown to be of no value in multivariate analysis (Lipponen *et al.*, 1994). In contrast, Shao and co-workers found a high apoptotic count to be associated with better prognosis in the 91 cases of breast cancer that they investigated (Shao *et al.*, 1996a).

A complete lack of correlation between apoptosis and prognosis has been described by two groups who investigated 82 cases of gastric cancer (Koshida *et al.*, 1997) and 148 cases of ovarian cancer of all histological types (Diebold *et al.*, 1996).

Thus apoptotic rate does not promise to be a good prognostic marker in most cancers. In many organs high apoptosis in cancer is associated with a trend towards worse prognosis. In some others it has no relation to prognosis at all. In lung cancer (NSCLC) on the whole there is a trend for high apoptosis to be associated with worse prognosis but in most series (including ours) this trend is not statistically significant. A study involving a larger number of patients might be able to give clearer answers.

Overall it is fair to comment that in most cancers apoptosis does not seem to have the effect on survival that is expected on theoretical grounds i.e. high apoptosis does not lead to better prognosis. There are several possible explanations:

Firstly, it may be that what is being measured as apoptosis in histological sections is inaccurate and therefore the conclusions derived from such measurements are likely to be false. If we could find a more reliable way of measuring apoptosis it would probably show that high apoptosis is associated with a better survival rate. Even without the contrary results mentioned above there have always been serious doubts about the

rationality of measuring a dynamic process like apoptosis in histological sections. As part of our investigations we performed some experiments to devise a better way of measuring apoptosis. The results of these experiments have been described in Section 5.5 and the implications of these results will be discussed later in Section 6.10.

Secondly, does apoptosis actually increase as tumours progress? If one believes that the present ways of measuring apoptosis are valid, this would be the inescapable conclusion from the clinical studies and the studies in pre-neoplasia mentioned in the previous section (Section 6.2). Such an increase could be a result of tumour expansion outstripping new vessel formation as discussed before (Section 6.2). It could also be a marker of disease progression. This would be contrary to our theoretical expectations but would certainly explain much of the empirical observations. We and several others before us have considered this question as we investigated the relation of apoptosis and other pathological markers of disease progression. The observations and results obtained from these various investigations will be discussed in the next section.

Thirdly, there remains the possibility that the rate of apoptosis does not actually change as the tumour progresses. The trend for shorter survival that has been noticed in some studies (including ours) is only a statistical aberration which will disappear when more studies are done with larger numbers of patients.

Fourthly, apoptosis might be important in some tumours but not in others.

6.4 Apoptosis and pathological markers

In our study we sought to determine whether there were correlations between apoptosis and other parameters of disease severity i.e. pathological stage, lymph node metastasis, histological differentiation or DNA ploidy. None was found (see Sections 5.3.2 to 5.3.6). Here we shall first discuss the correlation of stage and differentiation with apoptosis and then go on to discuss cell proliferation separately.

In their previously mentioned study, Stammers and Volm found no significant relation between apoptosis and disease stage or lymph node metastasis (Stammers and Volm, 1996). They had also investigated the relation between apoptosis and *in vitro* drug resistance assays, the findings of which have already been discussed in the previous section.

For their investigations Komaki *et al.* had selected only those patients of NSCLC who were pathologically staged as N1, therefore no inference about correlation with lymph node metastasis could be drawn from their study. They found no association between apoptosis and tumour size (T), grade of histological differentiation, location of tumour, age, performance status and weight loss of patients (Komaki *et al.*, 1996). Kargi *et al.* in their series only looked for possible correlation between apoptosis and disease stage and found none (Kargi *et al.*, 1997).

Tormanen *et al.*, who were the only group to find a prognostic significance for apoptosis in lung cancer, could not discern any association between the TNM status of a tumour and its apoptotic index, but the percentage of apoptotic cells was significantly higher in high grade carcinomas than in low grade carcinomas ($p = 0.019$).

A similar association between high apoptosis and poor histological differentiation has also been described in cervical carcinoma (Shoji *et al.*, 1996), ovarian carcinoma (Diebold *et al.*, 1996), prostatic adenocarcinoma (Vesalainen *et al.*, 1994), gastric carcinoma (Koshida *et al.*, 1997; Saegusa *et al.*, 1995c) and breast carcinoma (Lipponen *et al.*, 1994). A significant correlation in the opposite direction (i.e. higher apoptosis in well differentiated tumours) has been reported in breast cancer (Shao *et al.*, 1996a),

oesophageal carcinoma (Ohashi *et al.*, 1997). as well as in gastric carcinoma (Shinohara *et al.*, 1996) The latter could not find any correlation between apoptosis and disease stage.

In bladder cancer high apoptotic index has been found to correlate with several parameters of disease progression e.g. high pathological stage, high histological grade, more lymph node metastases, more distant metastases and DNA aneuploidy (Lipponen and Aaltoma, 1994).

In breast cancer Lipponen *et al.* found no correlation between apoptosis and tumour size (T) or lymph node status (N). They found high AI to correlate with poor histological differentiation (mentioned above), DNA aneuploidy and loss of oestrogen receptors (Lipponen *et al.*, 1994). The latter is a sign of advanced breast cancer and carries a poor prognosis. This association between apoptosis and loss of hormone receptor was also apparent strongly in another series (Frankfurt *et al.*, 1996). In contrast to this two groups have found that high apoptosis in breast cancers is associated with a significantly lower risk of lymph node metastasis (Sierra *et al.*, 1996; Shao *et al.*, 1996b).

Thus there seems to be no consistent association between apoptosis and other pathological parameters in the various tumours that have been investigated. In lung cancer only one group have found apoptosis to be associated with a marker of disease progression. In breast cancer there are conflicting reports by different groups. Even if one particular marker is selected (e.g. differentiation) it seems to have different associations with apoptosis in different tissues. Further investigations are called for before one can come to any firm conclusions. This is particularly true in the case of lung cancer where other than ourselves only one other group (Tormanen, 75 patients) have comprehensively investigated apoptosis against a range of pathological markers.

From the present evidence one would be tempted to draw the conclusion that while in some cancers increasing severity of disease is accompanied by increasing apoptosis, in lung cancer there does not seem to be any such correlation.

Several investigators have focussed on the relation between apoptosis and proliferation in tumours. Lipponen and Aaltoma were the first to report on a close correlation

between these two parameters in human epithelial malignancies. In a study involving 400 transitional cell bladder carcinomas they estimated cell proliferation from S phase fraction (SPF) and mitotic indices. They found a strong correlation between high AI and high proliferation rate ($p < 0.001$) (Lipponen and Aaltoma, 1994). Later that year they along with two other colleagues reported a similarly strong correlation between apoptosis and proliferation in 288 cases of breast cancer (Lipponen *et al.*, 1994). In both these series the cases with high AI had a shorter survival. After multivariate analysis the authors concluded that the prognostic value of AI in predicting survival was more likely to be related to cell proliferation than to cell death. Both processes were, after all, under common regulatory mechanisms. The oncogene c-myc is capable of causing both cell proliferation and apoptosis depending on whether the cell cycle is supported by growth factors or is inhibited by growth factor deprivation. Furthermore, cyclin dependent kinases normally stimulate proliferation, but if activated at an inappropriate time during the cell cycle they can cause apoptosis.

Following this two other studies in breast cancer (Samoszuk *et al.*, 1996; Frankfurt *et al.*, 1996) and one study involving 93 colonic carcinomas (Barreton) have found a strong direct correlation between apoptosis and proliferation.

In our series there was no correlation between AI and cell proliferation as assessed by AgNOR counts. Only two other groups have carried out similar investigations in lung cancer. Tormanen *et al* used PCNA to assess cell proliferation in their 75 lung cancers, while Eerola *et al* used Ki-67 staining for their 40 cases of SCLC. Neither of them found any statistically significant correlation between the two parameters. In gastric cancer a similar lack of correlation has been reported (Shinohara *et al.*, 1996).

An inverse correlation between apoptosis and proliferation has been reported in a series of 69 oesophageal squamous cell carcinomas (Ohbu *et al.*, 1995) and more recently in 92 skin neoplasms – both squamous cell and basal cell carcinoma (Shigihara and Lloyd, 1997).

Thus, like many other aspects of tumour biology, the relation between apoptosis and proliferation seems to depend on the type and site of the cancer.

In analysis of survival there was no difference in outcome between lung tumours with high AgNOR/low AI which are expected to accumulate cells much more quickly than those with low AgNOR/high AI (see Section 5.3.5). One could conclude that mere numerical superiority is not as important in tumour progression as the nature of the malignant cells that are being produced. Whether the cells are able to invade locally or metastasise to distant organs is likely to be a key determinant of outcome. Of course aberrant apoptosis could influence some of these characteristics as well.

6.5 Expression of bcl2 in lung cancers

The expression of bcl2 is associated in many systems with resistance to apoptosis, and so a primary aim of this work was to investigate bcl2 expression in a series of lung cancers and relate it to apoptosis and clinico-pathological parameters of disease severity. In our series of cases, 55.6% expressed bcl2 in at least some of their cells. 31.6% of the tumours were bcl2 positive i.e. more than 50% of the malignant cells had Bcl2 (see Section 5.4.1).

Although the expression and the pathological correlates of bcl2 had been studied extensively in lymphoid neoplasms, Francesco Pezzella and his co-workers were the first to demonstrate that bcl2 is expressed in epithelial malignancies as well (Pezzella *et al.*, 1993). In a series of 122 non small cell lung cancers (NSCLC) they reported bcl2 positivity in 20% of the whole group. Among squamous cell carcinomas (SCC) taken separately 25% were bcl2 positive while only 12% of the adenocarcinomas (AC) were positive. They also reported that bcl2 was important in predicting survival in certain subgroups of lung cancer. This caused considerable interest in the oncogene and in the last four years a vast body of literature has been published documenting the expression and role of bcl2 in carcinomas at different sites of the body. In lung cancer alone at the last count there were 15 major publications, each comprising more than 100 patients on an average, which have investigated the correlation of bcl2 expression with various pathological markers and with survival.

In the majority of publications on the subject, the incidence of bcl2 positive cases among NSCLCs taken as a whole has ranged between 20-35% (Higashiyama *et al.*, 1997; Kargi *et al.*, 1997; Ohsaki *et al.*, 1996; Ritter *et al.*, 1995; Tormanen *et al.*, 1995). Fontanini *et al.*, reported 67% of their 101 NSCLCs as bcl2 positive, but this is probably because they classified as positive any tumour where more than 1% of the cells stained positively with the monoclonal antibody (Fontanini *et al.*, 1995).

Among SCCs taken as a separate group the incidence of bcl2 positivity has been consistently reported between 25-35% (Jiang *et al.*, 1996; Ohsaki *et al.*, 1996; Oneill *et*

al., 1996; Pezzella *et al.*, 1993; Ritter *et al.*, 1995; Tormanen *et al.*, 1995; Volm and Mattern, 1996). Among ACs a much lower incidence has been generally observed – between 6-12% (Jiang *et al.*, 1996; Ohsaki *et al.*, 1996; Oneill *et al.*, 1996; Pezzella *et al.*, 1993; Tormanen *et al.*, 1995), but one group (Ritter *et al.*, 1995) have found a higher percentage of bcl2 positives among ACs (44%) than among SCCs (25%).

Among SCLCs a much larger fraction of tumours are bcl2 positive –65-90% in most studies (Benezra *et al.*, 1994; Jiang *et al.*, 1995; Jiang *et al.*, 1996; Kaiser *et al.*, 1996; Tormanen *et al.*, 1995; Yan *et al.*, 1996) and the lowest figure reported is 50% (Eerola *et al.*, 1997). Almost 100% of SCLC derived cell lines are bcl2 positive (Ikegaki *et al.*, 1994; Jiang *et al.*, 1996).

In squamous cell carcinoma of the head and neck three groups have recorded bcl2 positivity of between 17-36% of tumours (Friedman *et al.*, 1997; Gallo *et al.*, 1996; Wilson *et al.*, 1996). One of these groups (Gallo *et al.*) observed a suggestive association between tobacco exposure and bcl2 expression ($p < 0.1$). In oesophageal SCCs the bcl2 oncogene is overexpressed by a large fraction of tumours – between 45-60% in four large studies (Koide *et al.*, 1997; Ohbu *et al.*, 1997; Patel *et al.*, 1997; Puglisi *et al.*, 1996). Interestingly 100% of basaloid carcinomas of the oesophagus were bcl2 positive (Koide *et al.*, 1997). Among skin neoplasms too, 100% of BCCs are bcl2 positive compared to only 10% of SCCs (Moralesducret *et al.*, 1995; Verhaegh *et al.*, 1995).

In breast cancer, where the role of bcl2 has been examined minutely, several large studies have reported almost identical incidence figures (between 43-48%) (Hurlimann *et al.*, 1995; Joensuu *et al.*, 1994; Olopade *et al.*, 1997; Yamaguchi *et al.*, 1997), with only two reporting higher figures (62% and 65%) (Barbareschi *et al.*, 1996; Gasparini *et al.*, 1995). In glandular carcinomas elsewhere, substantial numbers of tumours express this gene e.g. 48% of prostatic adenocarcinomas (Apakama *et al.*, 1996), 37% of colonic cancers (Watson *et al.*, 1996), 18% of gastric cancers (Saegusa *et al.*, 1995b) and 57% of ovarian cancers (Herod *et al.*, 1996).

Thus, it can be seen that *bcl2* is overexpressed in a substantial number of cancers at different sites of the body. In lung cancer its expression seems to vary with histological type being almost universal among SCLC tumours and cell lines, present in a third of SCCs and in 10% of ACs. A closer inspection of the pattern of *bcl2* expression in various tumours will lead to some basic questions about the biology of NSCLCs.

(a) Are *bcl2* positive non-small cell lung cancers of neuroendocrine origin?

Just as the great majority of small cell lung cancers overexpress *bcl2*, they also commonly express various markers of neuroendocrine (NE) cells. Neuroendocrine (NE) cells are dispersed widely throughout the human body. Tumours arising from NE cells in different organs are histologically and cytologically diverse but share some common biochemical features which are used to identify these tumours. Some commonly used biochemical markers include L-dopa decarboxylase, neurone specific enolase and chromogranin. Such markers are present in more than 80% of SCLCs and in 10-30% of NSCLCs (Richardson and Johnson, 1993; Sundaresan *et al.*, 1991). Thus in small cell lung cancers the expression NE markers seems to go hand in hand with *bcl2* expression. Furthermore, one study in lung cancer showed *bcl2* overexpression to be associated with more aggressive neuroendocrine cell types (Coppola *et al.*, 1996) raising the possibility that *bcl2* might be a marker of neuroendocrine differentiation in these cells (Higashiyama *et al.*, 1995).

A similar intimate correlation between neuroendocrine differentiation and *bcl2* expression has been reported in prostatic carcinoma as well (Segal *et al.*, 1994). To investigate the situation among NSCLCs, Jiang *et al.* stained a series of lung cancer sections (SCC and AC) for both *bcl2* and NE markers. The majority of *bcl2* positive SCCs expressed multiple NE markers. Furthermore, *bcl2* positive cells could be roughly divided into those with neuroendocrine differentiation features which had intense *bcl2* staining and others which showed weak to moderate *Bcl2* staining. Thus *bcl2* appears to be closely associated with the neuroendocrine differentiation of lung tumour cells in general irrespective of whether they are NSCLCs or SCLCs. Jiang *et al.* have proposed that *bcl2* could be used as a NE marker in lung cancers (Jiang *et al.*, 1996).

The existence among NSCLCs of a separate group with NE features has been known for a long time and these tumours have been classified as NSCLC-NEs. These tumours are completely indistinguishable from their non-NE counterparts in every other aspect including histological appearance and their true identity may not be revealed until special tests are done. Jiang *et al.* were the first to demonstrate that bcl2 is commonly overexpressed by this group. If other investigators were to find a similarly strong correlation one may eventually have to agree with Jiang *et al.* that the bcl2 positive NSCLCs actually represent this separate biological entity. Since NSCLC-NEs are clinically known to behave in a distinct fashion it could explain the prognostic importance of bcl2 expression in non small cell lung cancer. This issue will be discussed again in Section 6.8.

(b) Are basaloid lung cancers more frequently found among bcl2 positive NSCLCs?

The other interesting feature to emerge from these statistics is that basal cell carcinomas (BCC) of the skin and oesophagus are almost universally bcl2 positive. These slow growing tumours arise from the basal cells of the epithelium, hence the name. As mentioned before, normal basal epithelial cells express bcl2 (see Section 1.3.3.2) and it seems that they maintain this original phenotype even after neoplastic transformation. Such bcl2 overexpression may contribute, in part, to the indolent nature of these skin neoplasms. BCCs have been shown to have a very low proliferative rate. Their growth is mainly dependent upon a slow apoptotic rate caused by excessive Bcl2. This type of neoplastic growth is possibly associated with less aggressive tumour behaviour.

Bcl2 positive NSCLCs too have been shown to be relatively indolent. Could it be that these tumours represent the pulmonary counterparts of cutaneous BCC? Recently a new morphologic and phenotypic entity called basaloid (basal cell) carcinoma of the lung has been described which is thought to arise from the basal bronchial epithelial stem cells. It can exist either in a pure form or equally often in association with SCC, AC or LC (large cell carcinoma) (Brambilla *et al.*, 1992). There needs to be a careful review of

histological sections of bcl2 positive NSCLCs to determine if basaloid carcinoma is more commonly found in association with NSCLCs which express this oncogene.

(c) At what stage of the cancerous process is bcl2 first seen to be overexpressed?

Whether or not bcl2 positive lung tumours are eventually proved to be basaloid cancers, there seems little doubt that most NSCLCs arise from the basal pluripotent reserve cells which are capable of differentiating along multiple lines. One of the earliest changes that is noticed in pre-malignant epithelium in the lungs and elsewhere is a change in the topographical location of bcl2 positive cells. From being confined to basal cells only in normal epithelium, bcl2 positive cells are found more and more frequently in the superficial layers as the lesions become more dysplastic (see Section 1.3.3.2). It can be argued that these initial changes in the location of bcl2 positive cells is an inevitable consequence of the structural disorganisation that accompanies dysplasia. Later, both the percentage of bcl2 positive cells as well as the intensity of staining continues to increase until in the most severely dysplastic lesions the entire thickness of the epithelium is often seen to be positively stained. This cannot be explained merely by structural reorganization and genetic mechanisms must be invoked. Reduced p53 expression can upregulate bcl2 in other tumours but in preneoplastic lung lesions, investigators have failed to find any correlation between the two (Walker *et al.*, 1995) and therefore it is unlikely to be the mechanism involved in lung neoplasms. An event primarily affecting the bcl2 gene is more likely. The nature of this molecular event is still unknown, but it is certainly not t(14:18) translocation. Whatever the cause of the overexpression bcl2 may serve to protect the growing pre-malignant lesion against excessive apoptosis and allow it to acquire yet more genetic mutations.

Oddly, during the step from severe dysplasia to frank malignancy in many tumours the expression of bcl2 seems to be downregulated. The best example of this was provided by Saegusa and coworkers who studied bcl2 expression in several grades of cervical intraepithelial neoplasia (CIN) and invasive squamous cell carcinoma (ISCC). Bcl2 immunoreactivity was seen in 37% of CIN grades I and II, 64% of CIN III and only 20% of ISCC (Saegusa *et al.*, 1995a). Nakamura too has recently reported a similar trend in

bcl2 expression in both gastric and colonic pre-neoplastic lesions (Nakamura *et al.*, 1997). Even in B cell lymphomas bcl2 expression is frequently lost during transformation from low grade into high grade lymphomas (Sander *et al.*, 1993). The paper of Walker *et al* provide indirect evidence that a similar terminal downregulation of bcl2 must take place in NSCLCs as well. In their series 48/56 dysplastic lung lesions (85%) strongly expressed bcl2 which was much higher than 14/31 carcinomas (45%) that expressed bcl2. From a teleological standpoint, does this indicate that protection from apoptosis is no longer an important factor once the threshold between pre-neoplasia and neoplasia has been crossed?

6.6 Apoptosis and bcl2

Bcl2 is an inhibitor of apoptosis and tumours that over-express bcl2 are expected to have a lower apoptotic rate. In our series of cases, however, this certainly was not the case, and if anything the opposite was true. High Bcl2 expression was associated with high apoptotic rate ($p = 0.041$, see Section 5.4.4).

Eerola *et al* have described similar results in their study involving 40 SCLCs. In their series bcl2 positive tumours exhibited higher apoptotic indices more often than bcl2 negative tumours ($p = 0.02$).

Tormanen *et al* found bcl2 positive NSCLCs to have a slightly lower apoptotic count but this was not statistically significant ($p = 0.13$). Several other investigators have found a complete lack of correlation between apoptosis and bcl2 in lung cancer (Hellquist *et al.*, 1997; Kargi *et al.*, 1997; Kennedy *et al.*, 1997; Oneill *et al.*, 1996). Only one group studying a subgroup of lung cancers with neuroendocrine (NE) features observed that bcl2 over expression, bax downregulation and particularly a bcl2/bax ratio of greater than 1 correlated with lower apoptotic indices ($p < 0.05$) (Brambilla *et al.*, 1996)

In cancers at other sites bcl2 overexpression is almost universally associated with a lower apoptotic rate. For example in breast cancer (Lipponen *et al.*, 1994; Frankfurt *et al.*, 1996; Sierra *et al.*, 1996) prostate cancer (Taniguchi *et al.*, 1996) colonic cancer (Baretton *et al.*, 1996) gastric cancer (Saegusa *et al.*, 1995b; Koshida *et al.*, 1997) a low apoptotic rate is always seen with bcl2 overexpression. Diebold and co-workers examining 148 cases of ovarian cancer found no correlation between bcl2 and apoptosis (Diebold *et al.*, 1996).

Thus while in cancers at almost all other sites bcl2 expression is seen to have its expected effect on the rate of apoptosis, lung cancer goes against theory and observation in *all* other cancers. This does need to be thought about carefully - the next paragraphs review possible explanations for what appears as a major anomaly.

(a) Is the Bcl2 that is overexpressed in lung cancer cells biologically inert?

In the laboratory specific point mutations can be engineered in the *bcl2* gene to render it biologically inert. Such mutations alter the BH1 and BH2 regions of BCL2 protein and prevent it from binding to Bax, its antagonist (Yin *et al.*, 1994; see also Section 1.3.2).

The possibility of such mutations taking place spontaneously in lung tumours has not been excluded. Theoretically, a point mutation in the gene might help explain another enigma surrounding Bcl2 expression. The molecular mechanism underlying *bcl2* overexpression in non-lymphoid tumours is not known yet. The t(14:18) translocation is not seen, neither is there any evidence of gene amplification (Reeve *et al.*, 1996). Point mutations in other genes, for instance p53, are known to stabilise the protein and increase its half-life within the cell, in addition to rendering it ineffective.

Although this theory is attractive, *in vitro* experiments seem to point against it. In SCLC cell lines Bcl2 protein levels (measured by Western blot analysis) were found to correlate with the amount of apoptosis. Treatment with antisense oligodeoxynucleotides targeting the *bcl2* coding sequence caused a dose dependent reduction of Bcl2 level and there was a parallel increase in apoptosis assessed morphologically (Ziegler *et al.*, 1997). Therefore the Bcl2 protein normally present in these lung cancer cells seem to be fully functional and critical for maintaining the viability of the cells *in vitro*. It is important to remember however that a necessity for Bcl2 for tumour cell survival *in vitro* does not guarantee a requirement for BCL2 *in vivo*.

(b) Is the protective effect of BCL2 negated by other factors?

Even though the Bcl2 molecule may be biologically active, its effects could still be overcome by its antagonists within the cell. NSCLC cell lines have been shown to express both anti-apoptotic proteins like Bcl2 and Bcl-xL and death accelerator proteins such as Bax and Bcl-xS (Reeve *et al.*, 1996). As mentioned before, these proteins can dimerise with each other and cancel out each others effects (see Section 1.3.4). An excess of Bax would neutralise the anti-apoptotic effect of Bcl2 and make the cell more prone to apoptosis. The relative ratio of the death suppressors and death promoters rather than the absolute amount of an individual protein may be more predictive of cellular susceptibility to apoptosis. It is not surprising therefore that Brambilla *et al* in

their study involving 121 neuroendocrine (NE) lung tumours found a Bcl2/Bax ratio > 1 to correlate with a lower apoptotic index while most other studies investigating Bcl2 alone found no such correlation. Recently Apolinario *et al.* have evaluated bcl2 and bax expression in histological sections of a series of 121 NSCLCs, but, unfortunately they did not quantify apoptosis (Apolinario *et al.*, 1997). Further studies, perhaps including a larger panel of Bcl2 family members, are imperative to unravel the way these proteins control apoptosis in cancer tissues *in vivo*.

(c) Does apoptosis in lung tumours take place by Bcl2 independent pathways?

Not all forms of apoptosis can be blocked by bcl2. A notable example is the apoptosis caused by cytotoxic T lymphocytes (CTL) (Vaux *et al.*, 1992).

There is abundant evidence that lymphocytes play a central role in the host response to tumours. (For a review of the subject see Graubert and Ley, 1996). Lymphocytes kill target cells (including tumour cells) by inducing them to undergo apoptosis. The granzymes (gzmA and gzmB) are a family of neutral serine proteases that are critical for this process. Bcl2 fails to prevent apoptosis in targets of such cell mediated killing.

Lymphocytic infiltration is commonly observed in lung cancers as in other cancers and lymphocytes are also often seen in very close association with apoptotic cells. It may be that *in vivo* lymphocytes provide a major stimulus for apoptosis in lung cancers which is why this process appears to be independent of the amount of Bcl2 expressed by the cells.

6.7 Bcl2 and clinico-pathological features

In order to investigate the role of bcl2 expression in lung cancer progression we examined the clinico-pathological features of bcl2 positive tumours to see if pathologically they represented a less aggressive group. Among our 134 patients we could discern no significant association between bcl2 expression and disease stage or lymph node metastasis (Section 5.4.6). There was a non-significant trend for poorly differentiated tumours to have higher bcl2 indices (Section 5.4.7). The majority of other investigators in lung cancer have reported similar results i.e. they could find no correlation between bcl2 expression and tumour size (T), nodal status (N) or histological grade (Fontanini *et al.*, 1995; Kargi *et al.*, 1997; Pezzella *et al.*, 1993; Tormanen *et al.*, 1995; Yan *et al.*, 1996). Two groups reported more bcl2 positive cases in stages I and II than in the higher stages (Higashiyama *et al.*, 1997; Ohsaki *et al.*, 1996).

We also investigated some of the non-traditional markers of disease progression against bcl2 expression (Section 5.4.7). In many tumours at other sites (see below) bcl2 overexpression has been found to be closely associated with a slower proliferation rate. In our series there was no association between bcl2 and proliferation rate as assessed by AgNOR. Other investigators who have used PCNA, Ki-67 and mitotic indices to assess proliferation rate in lung cancer, have also reported a lack of correlation (Fontanini *et al.*, 1995; Kaiser *et al.*, 1996; O'Neill *et al.*, 1996). One group found a significantly higher proliferation rate (assessed by Thymidine labelling) in the bcl2 positive lung tumours (Kennedy *et al.*, 1997).

There was a non-significant trend for higher bcl2 counts in aneuploid tumours in our series ($p = 0.160$, see Section 5.4.7). We could find no other study in lung cancer which had investigated these two parameters together. Joensuu *et al.*, investigating breast cancer, found bcl2 to be strongly correlated with DNA diploidy (Joensuu *et al.*, 1994).

In the recent past, there has been considerable interest in the role of neoangiogenesis in tumour progression and metastasis. Two recent studies which have investigated this

parameter in NSCLCs found *bcl2* not to influence the rate of new vessel formation in lung tumours (Apolinario *et al.*, 1997; Pastorino *et al.*, 1997).

The association of *bcl2* with other oncogenes in lung cancer is another area of very active interest. Our findings about the correlation between *bcl2* and CD40 are detailed in Section 5.4.3. Most other investigators, both in lung cancer and in cancers elsewhere have concentrated on the association between *bcl2* and p53. Normally, p53 downregulates the expression of *bcl2* and upregulates its antagonist bax. It thus alters the *bcl2*/bax ratio in the cell in favour of apoptosis (see Section 1.2.3.5 b). If this regulatory mechanism is maintained in cancerous tissues, one would expect to see an inverse correlation between the two oncogenes.

This however is manifestly not the case in lung cancers as shown by the investigations of several groups who found no correlation between the two (Eerola *et al.*, 1997; Kennedy *et al.*, 1997; Ohsaki *et al.*, 1996; Reeve *et al.*, 1996; Tormanen *et al.*, 1995; Yan *et al.*, 1996). As mentioned before even in pre-malignant lung lesions, *bcl2* and p53 are found not to be correlated. Thus in lung cancer cells the *bcl2* gene seems to be independent of its normal regulatory control. In breast cancer, however, this is certainly not the case and investigators have invariably found an inverse correlation between *bcl2* and p53 (Barbareschi *et al.*, 1996; Gasparini *et al.*, 1995; Hurlimann *et al.*, 1995; Joensuu *et al.*, 1994; Olopade *et al.*, 1997; Silvestrini *et al.*, 1994). In colonic carcinoma (Watson *et al.*, 1996; Kaklamanis *et al.*, 1996) and gastric cancer (Saegusa *et al.*, 1995b) too the inverse correlation is maintained.

In lung cancer the association between *bcl2* and chemoresistance is complex. Small cell lung cancers, although they are *bcl2* positive, usually show a good response to chemotherapy whereas in NSCLCs the opposite is usually the case. Volm and Mattern found *bcl2* positive lung SCCs to be invariably resistant to doxorubicin in an *in vitro* test. In their series of 85 patients *bcl2* overexpression was strongly related to the resistance related proteins P-glycoprotein and glutathione S-transferase (Volm and Mattern, 1995). Wu and El-diery found no such correlation between *bcl2* and chemoresistance in four lung cancer derived cell lines. However they found that induction of apoptosis correlated very strongly with chemosensitivity irrespective of *bcl2* and p53 status (Wu and El-diery,

1996). Transfection of *bcl2* into the SCLC derived cell line SBC-3 increased its resistance to some chemotherapeutic agents but not to others (Ohmori *et al.*, 1993) indicating that there are *bcl2* independent pathways of apoptosis in lung cancer cells. Overall, *bcl2* status does not promise to be a good guide for predicting response to neo-adjuvant chemotherapy in lung cancer.

In breast cancer *bcl2* expression has been consistently demonstrated to be associated with favourable clinico-pathological factors. The majority of investigators have found *bcl2* to be associated with smaller tumour size (T) and lower stage (Barbareschi *et al.*, 1996; Kobayashi *et al.*, 1997; Sundblad *et al.*, 1996; Olopade *et al.*, 1997; Silvestrini *et al.*, 1994 the sole exception being Joensuu *et al.* who found no such correlation. In general *bcl2* does not seem to affect the lymph node status (N) (Kobayashi *et al.*, 1997; Joensuu *et al.*, 1994; Yamaguchi *et al.*, 1997) although in one study *bcl2* positive breast tumours seemed to have a higher chance of lymph node disease (Sierra *et al.*, 1996). Without exception *bcl2* has been found to be associated with better differentiated tumours, and with oestrogen receptor positivity prompting some authors to comment that *bcl2* may be a differentiation inducing factor in breast cancer cells. Because of all these favourable associations of *bcl2* in breast cancer it comes as no surprise that *bcl2* positive breast cancers should fare so much better than their *bcl2* negative counterparts (see Section 6.8).

Contrariwise, in prostate cancer *bcl2* expression is associated with unfavourable clinico-pathological parameters e.g. large tumour size (Bubendorf *et al.*, 1996), poor differentiation (Stattin *et al.*, 1996) and androgen independent phenotype (McDonnell *et al.*, 1992). *Bcl2* positive prostatic cancers have been found to respond poorly to hormone therapy (Apakama *et al.*, 1996; Taniguchi *et al.*, 1996) which is understandable since hormone ablation supposedly acts by causing apoptosis, something which is less likely to happen if there is plenty of *bcl2* protein within the cell. Strangely, however, in breast cancer radiotherapy, chemotherapy and anti-hormone therapy (Tamoxifen) all three of which are known to act by causing apoptosis, are invariably more effective in *bcl2* positive tumours (Elledge *et al.*, 1997; Gee *et al.*, 1994; Willsher *et al.*, 1996).

In summary , it can be said that for the three cancers we have examined here - lung, breast and prostate - bcl2 expression is not correlated with clinico-pathological features (lung); correlated with favourable features (breast); and with adverse features (prostate). In the next section the correlation of bcl2 with survival will be reviewed in the light of its associations (positive or negative) with apoptosis and clinico-pathological features.

6.8 Bcl2 and survival

Bcl2 overexpression, by preventing apoptotic cell death, is expected to lead to a rapid increase in tumour size and therefore a worse prognosis. In practice the opposite is often found to take place in lung cancers. In our study patients were followed up for 10 years following resection of their lung tumours. Those who were bcl2 positive lived longer than those who were bcl2 negative ($p = 0.013$, see Section 5.4.5). Similar results have been reported in a number of other cancers, and this apparent paradox needs explanation.

Pezzella *et al.* were the first to report the beneficial effect of bcl2 expression in lung cancers (Pezzella *et al.*, 1993). In their 122 cases of NSCLC taken together, they found no correlation between bcl2 and survival. But when the 80 SCCs were considered separately, the bcl2 positive tumours were found to give a better chance of survival over a five year period ($p < 0.05$). In this group bcl2 proved a better predictor of prognosis than the N-status of a tumour.

Better prognosis for bcl2 positive SCCs has also been reported by two other groups (Ritter *et al.*, 1995; Volm and Mattern, 1995). Other investigators have found bcl2 expression to have a beneficial effect in all NSCLCs irrespective of the histological subtype (Apolinario *et al.*, 1997; Fontanini *et al.*, 1995; Ohsaki *et al.*, 1996; Pastorino *et al.*, 1997). In SCLCs as well, Kaiser and coworkers have reported a trend towards longer survival in bcl2 positive tumours (Kaiser *et al.*, 1996). Two groups have reported a lack of correlation in lung cancer (Oneill *et al.*, 1996; Tormanen *et al.*, 1995).

The effect of bcl2 expression has been studied in many other cancers. In breast cancer the advantage of bcl2 expression was first demonstrated by Silvestrini *et al.* and subsequently corroborated by several others (Silvestrini *et al.*, 1994; Elledge *et al.*, 1997; Hurlimann *et al.*, 1995; Joensuu *et al.*, 1994; Kobayashi *et al.*, 1997; Lipponen *et al.*, 1995; Yamaguchi *et al.*, 1997). Other tumours which show a similar positive correlation include ovarian cancer (Diebold *et al.*, 1996; Herod *et al.*, 1996; Marx *et al.*, 1997) and colonic cancer (Baretton *et al.*, 1996; Ofner *et al.*, 1995). However in colonic cancer some investigators have found no correlation between bcl2 and survival (Pereira *et al.*,

1997; Schneider *et al.*, 1997; Scott *et al.*, 1996). Squamous cell carcinomas of the oesophagus seem to progress independently of bcl2 expression (Koide *et al.*, 1997; Puglisi *et al.*, 1996) but SCCs of the head and neck fare worse if they are bcl2 positive (Friedman *et al.*, 1997).

In prostate cancer bcl2 positive tumours have been found to have a worse prognosis (Bauer *et al.*, 1996; Bubendorf *et al.*, 1996). Similarly in leukaemias and lymphomas bcl2 positive cases usually have a shorter survival.

If we integrate the observations in this section with that in the previous two (Sections 6.6 and 6.7) it will be clear that the effect of bcl2 expression on apoptosis and clinico-pathological status is highly variable among tumours. As an example the observations in three common tumours are summarised in Table 6.1.

Table 6.1 **Correlation of bcl2 expression with apoptosis and clinico-pathological parameters in three common tumours.**

Cancer	Increased bcl2 expression is associated with			
	Apoptosis	Differentiation	Size (T)	Survival
Breast	decreased	better	smaller	better
Prostate	decreased	worse	larger	worse
Lung	no association	no association	no association	better

In bcl2 positive breast cancer, survival is prolonged and this could be because these tumours are better differentiated and of smaller size. A lower rate of apoptosis in these tumours does not seem to off-set the advantage conferred by the other two factors. Bcl2 positive prostate cancers on the other hand are larger, less well differentiated and show less apoptosis and not surprisingly they die earlier. In the great majority of cases of non-small cell lung cancer, bcl2 expression is associated with prolonged survival. Why this should happen is not clear but some possible reasons will be discussed below.

When Pezzella *et al.* first reported in 1993 that bcl2 positive lung cancers survive longer, they ascribed the beneficial effects to the ability of bcl2 to prevent apoptosis.

Because *bcl2* positive tumours were expected to have a slower apoptotic rate, they would be able to survive and grow even if the rate of cell division were low. A low mitotic rate would lead to a slower rate of acquisition of additional genetic defects as suggested by McDonnell *et al.* from observations in transgenic mice (McDonnell *et al.*, 1989). Less genetic defects would make for a more indolent phenotype and therefore longer survival. To prove their point Pezzella *et al* cited the example of follicular lymphomas where *bcl2* expression is a frequent primary aberration. These tumours have a slow apoptotic rate, a low mitotic rate and an excellent prognosis presumably because they acquire fewer additional mutations (Vaux *et al*, 1988).

For a time this seemed a very plausible explanation until two of the basic assumptions made by Pezzella were proved to be wrong by subsequent investigations. *Bcl2* positive lung tumours neither have a slow apoptotic rate, nor do they have a lower rate of cell proliferation. Although in other tumours *bcl2* expression does reduce the rate of apoptosis, in lung cancer this is not found to happen. The reasons for this are explained in Section 6.6. Also, *bcl2* positive tumours proliferate just as rapidly as their *bcl2* negative counterparts and therefore they should have just the same risk of acquiring additional genetic defects. In practice the vast majority of *bcl2* positive tumours do not show any pathological indication (i.e. T and N stage) of being more indolent yet they survive longer.

Soon afterwards, in 1994, Pietenpol *et al.* reported some unexpected experimental observations which could explain the prognostic effects of *bcl2*. They demonstrated that *in vitro* *bcl2* overexpression could suppress the growth of several solid tumour cell lines including a lung adenocarcinoma cell line H1299. This was surprising because *bcl2* had been shown previously to stimulate the growth of other cell types under circumstances otherwise leading to apoptosis. Pietenpol's observation seemed to indicate that in certain tissues *bcl2* may act as a tumour suppressor gene (Pietenpol *et al.*, 1994). It is not unusual for oncogenes and growth factors to have widely disparate effects on different cell types depending on lineage and stage of differentiation. For instance introduction of *ras* genes or proteins into certain cell lines induces differentiation rather than

proliferation and in some cases this is accompanied by growth arrest (Nakagawa *et al.*, 1987). Expression of *fos* has been shown to induce growth arrest in tumour derived cell lines (Garrido *et al.*, 1993).

However, effects observed *in vitro* are not always paralleled by *in vivo* observations. Breast cancers overexpressing *bcl2* are usually smaller in size which is what one would expect following Pietenpol's experiments. In lung cancer, though, most groups have found no correlation between tumour size (T) and *bcl2* status.

For an explanation it may be necessary to look beyond *bcl2*'s role in tumour growth rate and focus on some other putative aspect of its biology. As noted in Section 6.7, *bcl2* is associated in some cancers with differentiation status of the tumour, and in 1995 Hockenberry had postulated that the *bcl2* gene may also have a role in cell differentiation (Hockenberry, 1995). During their life span immature cells pass through sequential transitions to a differentiated state and eventually undergo cell death and in parallel with this, *bcl2* expression usually decreases as cells become more differentiated. In *in vitro* experiments, when cell lines derived from myeloid precursors are induced to differentiate with a variety of agents (retinoic acid, phorbol ester, interleukin-6) the expression of *bcl2* is observed to be concomitantly down regulated (Benito *et al.*, 1995; Nagy *et al.*, 1996). The question arises - is this down regulation a cause or a consequence of cell differentiation? In HL-60 cells, Blagosklonny *et al.*, have shown that the actual loss of Bcl2 protein is not required for the accomplishment of the differentiation programme and the terminal loss of *bcl2* expression is just one of the many changes that take place in differentiating cells (Blagosklonny *et al.*, 1996). In epithelial cells however the situation may be slightly different. Transfection of *bcl2* into primary human keratinocyte derived cell lines not only prevents apoptosis but also blocks further differentiation leading one to conclude that *bcl2* influences epidermal differentiation in addition to blocking apoptosis (Nataraj *et al.*, 1994; Marthinuss *et al.*, 1995). In neural differentiation *bcl2* expression increases as cells become more differentiated (mature nerve cells are irreplaceable and cannot be allowed to die). If the over-expression of *bcl2* in immature

cells is blocked by anti-sense oligonucleotides no further differentiation occurs (Zhang *et al.*, 1996).

In breast cancer bcl2 positive tumours are invariably well differentiated and oestrogen receptor positive, suggesting that bcl2 may indeed be related to tumour differentiation at this site (although in a manner opposite to that in keratinocytes and more akin to neural cells). However in non-small cell lung cancer there is no correlation between bcl2 and histological differentiation (see Section 5.4.7).

It may be that in lung cancer the bcl2 gene induces cells to differentiate along particular histological lines. The possibility of NE tumours and basaloid lung cancer has already been discussed (see Section 6.5). Initial small studies have shown a strong correlation between bcl2 overexpression and NE differentiation of lung cancers. The NSCLC-NEs represent a relatively benign group of tumours which are more likely to respond to chemotherapy (Linnoila *et al.*, 1989). However long term studies have shown that despite a better initial response the patients did not live a longer period of time (Sunderesan *et al.*, 1991). Similarly the basaloid hypothesis even if proved to be true may not explain the prognostic advantage of bcl2. Basaloid lung cancers, unlike their skin counterpart, run an aggressive course leading to a poor prognosis. The median survival rate is only 22 months for Stage I and II disease (Brambilla *et al.*, 1992).

For an explanation many have turned to bcl2's inverse correlation with p53. Mutation of this tumour suppressor gene leads to loss of function and also to overexpression of the protein product due to an increase in its half life. Mutation of p53 occurs in about 60% of human malignancies and very commonly leads to poor prognosis. The mutant p53 protein is still able to downregulate bcl2 expression and therefore maintains some aspects of its normal physiology. (Haldar *et al.*, 1994) An inverse correlation between bcl2 and p53 is therefore a very common observation in many malignancies.

It has been argued that the sole reason that bcl2 positive tumours fare better is because they are associated with normal p53 function. Again this may be true in some cases but not in the case of NSCLC where the normal physiological control mechanisms between bcl2 and p53 seems to be disrupted from a very early stage in tumourigenesis. Walker *et*

al found bcl2 expression in dysplastic lung lesions to be independent of p53 expression (Walker *et al.*, 1995). In lung cancer cell lines Reeve *et al* could find no correlation between the two proteins (Reeve *et al.*, 1996). In keeping with these observations, the vast majority of studies involving lung cancer sections have found no correlation between bcl2 and p53.

Having thus exhausted the more likely explanations it may now be necessary to look for possible associations of bcl2 against a wider array of pathological markers e.g. new vessel formation, matrix metalloproteinase synthesis etc. Several oncogenes have been shown to have initially unexpected biological effects in tissues. Such investigations might prove valuable for two reasons. Firstly, by placing it on a firm theoretical footing it would strengthen the case for routinely using bcl2 status as a prognostic indicator in NSCLC. Secondly, the results of such investigations might provide new insight into the pathological and molecular mechanisms of lung cancer and thus indicate new ways of tackling it.

6.9 CD40 expression in squamous cell cancer of the lung

A major part of our investigations involved a systematic analysis of the pathological and biological associations of CD40 expression in squamous cell carcinoma of lung (see Section 1.4). In our series 22% of the SCCs were CD40 positive (i.e. more than 50% of the tumour cells stained positively). 41% of the SCCs expressed CD40 in at least some of their cells (Section 5.4.2). 3 of the 6 adenocarcinomas in our series of fresh lung tumours were CD40 positive (see Section 5.5.2.6).

On a ten year follow up, there was no difference in survival between the CD40 positive and CD40 negative groups, even when differences in stage, grade, proliferative rate and ploidy were corrected for.

On analysis of the pathological markers, CD40 expression did not correlate with tumour stage, lymph node metastasis, histological grade or proliferative index as assessed by AgNOR. There was a significant correlation between tumour ploidy and CD40 status. Diploid tumours had higher CD40 indices compared to aneuploid tumours ($p = 0.029$).

In an extensive survey of literature we could find only one other study attempting to correlate CD40 expression with clinico-pathological features of cancer. Vandenoord *et al* analysed the role of CD40 as a prognostic marker in 71 cases of malignant melanoma (MM). They found CD40 to be a bad prognostic factor in this skin neoplasm (Vandenoord *et al.*, 1996). Like many other cancers, MMs are known to progress step-wise through various pathological stages i.e. melanocytic naevus, dysplastic naevus, radial growth phase (RGP), vertical growth phase (VGP) and finally metastatic melanoma.

In their series Vandenoord *et al.* found CD40 to be expressed in a larger fraction of the VGP melanomas than in the RGP melanomas. Patients expressing CD40 in the vertical growth phase of their melanomas did not differ significantly from CD40 negative patients with respect to any of the known prognostic parameters but showed a significantly shorter tumour free survival. In about a third of the VGP tumours, CD40-L was seen in

the same tumour areas that expressed CD40. Patients with CD40(+)/CD40-L(+) malignant melanomas tended to have a shorter tumour free survival than those lacking CD40-L. These findings suggest that the CD40/CD40-L autocrine growth loop is an important disease modifying factor in the vertical growth phase of MM.

A significant shortcoming of our investigations was the lack of information about CD40-L expression among the lymphocytes that were frequently seen to infiltrate the tumour areas. This limitation makes it difficult to interpret the results of some of the analyses that we undertook i.e the effect of CD40 on apoptosis and the inter-relation of CD40 and bcl2 expression.

In our series there was no correlation between CD40 expression and the apoptotic index of a tumour (Section 5.4.8) but this result must be interpreted with caution. One cannot be certain if the CD40 that is seen to be expressed on the cell surface is likely to have been activated by the presence of CD40-L bearing cells in the vicinity. Ideally one would have to stain for both CD40 and CD40-L and compare the apoptotic indices of CD40(+)/CD40-L(+) tumours with that of CD40(-)/CD40-L(-) and CD40(+)/CD40-L(-) tumours. Even on theoretical grounds it is difficult to predict the outcome of such an analysis as the *in vitro* evidence is scarce and conflicting. CD40 activation has been seen to variously promote and prevent apoptosis in normal and transformed epithelial cells (see Section 1.4.4.3). The situation is uncertain and it may be that CD40 has different effects on different types of epithelium. Such uncertainty makes it all the more imperative to conduct detailed analyses.

Uncertainty also surrounds the relationship between CD40 and the bcl2 family of genes. In situations where it prevents apoptosis (e.g. in B cells) activation of CD40 has been seen to upregulate the expression of either Bcl2 or Bcl-x_L depending on the cell line that is being investigated (see Section 1.4.5.3). Regarding epithelial cell lines, we could find no information in the literature about the relation between CD40 and the bcl2 family.

Our investigations indicate a trend towards inverse correlation between CD40 and bcl2 expression in squamous cell carcinoma of lung (see Section 5.4.3). The implications of this inverse correlation are not immediately clear. Lack of information about CD40-L expression is a major handicap to meaningful interpretation of the results. The mean

apoptotic index of the CD40(+)/Bcl2(-) group was lower than the mean AI of the CD40(+)/Bcl2(+) group but this difference was not statistically significant. Neither was there any significant difference between the AIs of the other groups created according to their Bcl2 and CD40 status (see Section 5.4.12). The immediate impression is that the co-expression of these two oncogenes does not seem to significantly alter the course of apoptosis in squamous cell carcinoma of lung. Neither does it influence any other pathological marker e.g. stage, grade, lymph node status, ploidy or differentiation. When survival time was investigated Bcl2(+)/CD40(-) patients survived the longest (66.9 months) compared to Bcl2(-) /CD40(-) patients (29.7months). There was no difference between CD40(+)/Bcl2(-) and CD40(+)/Bcl2(+) as regards survival.

From these initial observations it does not seem likely that CD40 will emerge as an important prognostic indicator in SCC even when it is considered in conjunction with Bcl2 expression. Concomitant examination of the expression of CD40-L may reveal significant pathological and clinical associations. Further investigations are necessary.

6.10 Measurement of apoptosis in cultured tumour cells

There have always been doubts about the rationality of measuring a dynamic process like apoptosis in a static system like a histological section. No matter how refined the staining technique employed, from a section one can never properly estimate the duration of apoptosis which is one of the key determinants of tissue turnover kinetics. This is an important lapse as, both the speed of formation of apoptotic bodies and the speed of phagocytosis are known to vary widely from tissue to tissue, and small variations can result in large overall differences in apoptotic index.

Tormanen *et al.* in their investigations attempted to estimate the duration of apoptosis in tumour tissues on the basis of the relative occurrence of various types of apoptotic bodies thought to represent the various stages of apoptosis (Tormanen *et al.*, 1995). In their study, the ratio between apoptotic cells and bodies in lung carcinoma was roughly 1:2, whereas in control lung tissue it was 1:1. This was taken to indicate a higher turnover rate of apoptosis in tumours when compared to non-neoplastic tissues. However, the correction they suggested in the calculations can at best result in a crude estimate of the rate of apoptosis.

By definition apoptotic rate is the rate of change of apoptosis with time. To calculate such a rate one would need two estimations of apoptosis separated by a measured interval of time. Obviously a histological section would be singularly inadequate for this purpose. A suspension of live cells obtained from fresh disaggregated tumour tissue would be needed from which samples could be drawn at intervals. The rate of rise of the fraction of apoptotic cells from one sample to the next would theoretically give the exact rate of apoptosis. This is the principle on which we based a series of experiments conducted on pleural effusions and fresh lung tumours (see Section 4.2). The aim of our experiments was to investigate if the apoptotic rate of a tumour calculated in this fashion correlated with the apoptotic index obtained from the corresponding histological section.

Before considering this question, let us first examine the validity of the results obtained from the cell suspensions. There are several sources of error which we have not been

able to eliminate completely from our calculations. One main worry is that mechanical/enzymatic disaggregation to which the tumours were subjected could probably cause apoptosis or morphological changes resembling apoptosis and thus potentially distort the results to a significant degree. To avoid this source of distortion we initially conducted our experiments on pleural effusions. Here the malignant cells are already in suspension and there would be no need for disaggregation of any kind. Previously, in our laboratory, similar experiments have been done on leukaemia cells (Chant *et al.*, 1996). The pleural effusions would serve as an intermediate step between leukaemia and solid cancers (i.e. lung cancer). Unfortunately the experiments on pleural effusions proved to be unsuccessful for other reasons (see Section 5.5.1).

To eliminate this source of error from the results obtained from fresh lung tumours we adopted a subtractive approach as outlined in Section 5.5.2.1. The rationale behind this approach is that disaggregation might distort $Ao(\text{Day}0)$ but would leave $Ao(\text{Day}1)$ unaffected. Subtracting Day0 from Day1 would eliminate this error and give a true picture of the amount of apoptosis taking place *in vitro* in 24 hours. But a small source of error would probably still remain. Before estimating apoptosis on Day1 adherent cells were removed from the culture flasks by treating with Trypsin for 5 minutes. This is as likely to cause artefactual apoptosis as treatment with other enzymes (e.g. hyaluronidase, collagenase) which were used for disaggregation. Thus, $Ao(\text{Day}1)$ is probably prone to the same distortions as $Ao(\text{Day}0)$ but to a lesser degree.

Ideally one would want to separate the viable cells from the apoptotic cells immediately after disaggregation so that the starting cultures had no apoptotic cells at all. This way one would ensure that the apoptosis observed at 24 hours was genuinely the result of apoptosis *in vitro*. The technicalities of implementing such a cell separation are still being worked out in our laboratory.

Bearing in mind such reservations, if one were to embark on a comparison between the apoptotic rate and the apoptotic index obtained from histological sections, the first striking feature is that apoptosis in cultured cells is much higher than apoptosis in histological sections however measured. This is not totally unexpected because the cultured cells are deprived of several survival signals which are normally present *in vivo*.

Close contact with neighbouring cells and the extracellular matrix (ECM) is known to send strong anti-apoptotic signals via surface integrin receptors (Section 1.2.3.2). Paracrine and endocrine growth factors are also known to play an important role. The cells in culture are deprived of such survival signals and therefore undergo apoptosis at a much faster rate. Freed of many of the usual outside influences, this could be thought to represent an “intrinsic” apoptotic rate which is at least useful in comparing between tumours. One may argue that the circumstances under which this “intrinsic” rate is being obtained are so far removed from reality as to be of no practical relevance whatsoever. It is certainly true that the situation in the tumour nodules *in vivo* does not resemble that in the culture flasks and hence the total lack of correlation between the AIs obtained from histological sections and those obtained from cell culture. However, even *in vivo*, there are situations when the tumour cells are free of the influence of close contact with other cells or ECM e.g. metastatic cells in the blood circulation. Such cells are likely to have high apoptotic rates comparable to that seen in culture flasks.

Thus the cultured cells might tell us of the state of affairs prevailing during a very important phase of the tumours natural history (i.e. metastasis) which often determines clinical outcome. It is possible, therefore, that the apoptotic rate obtained from the cultured cells might give a somewhat better indication of prognosis than AI obtained from sections. The latter as we have seen is of very limited prognostic value in most cancers and is of almost no value in lung cancer. Thus, it would be extremely useful to calculate the apoptotic rates from fresh specimens of a larger number of patients of lung cancer who should be followed up prospectively to monitor disease outcome.

6.11 Concluding remarks

Several facts emerge from our investigations. Apoptotic index obtained from histological sections is of very little prognostic value in most cancers including lung cancer. And yet one intuitively feels that apoptosis - which does seem to vary widely from tumour to tumour - must have some influence on the outcome of cancer. Are we measuring apoptosis properly? In our cell culture experiments (discussed in Section 6.10) we have tried to formulate an alternative way of measuring apoptotic rate which we believe is more realistic and therefore more likely to correlate with clinical outcome. Of course the method we employed is cumbersome and requires fresh tissue as its raw material which is a distinct disadvantage. Also, some procedural refinements are necessary. For instance it might be a better idea to measure apoptosis at intervals of 8 hours or 16 hours rather than 24 hours, to prevent secondary necrosis of apoptotic cells.

Whether apoptosis measured in this fashion correlates with clinical outcome remains to be seen from prospective studies which would logically be the next step in the investigations. It is possible that it might still not emerge as a relevant factor - and thereby confirm the suspicion that apoptosis is not of very great import in an established tumour although it is almost certainly important during carcinogenesis.

A great deal more needs to be learnt about the genes controlling apoptosis. For instance, *bcl2* probably regulates epithelial cell differentiation in addition to blocking apoptosis (see Section 6.8). It may influence other physiological processes as well depending on cell lineage. The effect on one process (e.g. differentiation) may confound the effect on another (e.g. apoptosis) and hence the unexpected (in our present state of knowledge) observation that *bcl2* prolongs survival in lung cancer.

Knowledge about CD40 in lung cancer is truly very, very limited. Our investigations have shown that a substantial fraction of lung cancers express this surface molecule but this does not seem to influence matters (apoptosis, clinico-pathological parameters or survival) to any appreciable degree. The question of co-operation between *bcl2* and CD40 still remains unresolved.

Thus, at the end of our investigations, we have answered some questions but many more new ones have emerged. This confirms the old adage that the more we know, the more we discover that we don't know.

APPENDICES

APPENDIX A

WHO II Lung Cancer Classification of 1982

I. Epithelial Tumours

- A. Benign
 - 1. Papillomas
 - 2. Adenomas
- B. Dysplasia/ carcinoma in situ
- C. Malignant
 - 1. Squamous cell carcinoma
 - a. Spindle-cell variant
 - 2. Small cell carcinoma
 - b. Oat cell carcinoma
 - c. Intermediate cell type
 - d. Combined oat cell carcinoma
 - 3. Adenocarcinoma
 - c. Acinar
 - d. Papillary
 - e. Bronchioloalveolar
 - f. Solid carcinoma with mucin formation
 - 4. Large cell carcinoma
 - d. Giant cell carcinoma
 - e. Clear cell carcinoma
 - 2. Adenosquamous carcinoma
 - 3. Carcinoid tumour
 - 4. Bronchial gland carcinoma
 - 5. Others

II. Soft Tissue Tumours

III. Mesothelial Tumours

- A. Benign
- B. Malignant

I. Miscellaneous Tumours

- D. Benign
- E. Malignant

I. Secondary Tumours

II. Unclassified Tumours

III. Tumour-like lesions.

APPENDIX B

TNM classification of lung cancer

PRIMARY TUMOUR (T)

- T0** No evidence of primary tumour
- TX** Tumour proven by the presence of malignant cells in bronchopulmonary secretions but not visualised roentgenographically or bronchoscopically, or any tumour that cannot be assessed as in a retreatment staging.
- TIS** Carcinoma in situ
- T1** A tumour that is 3.0 cm or less in greatest dimension surrounded by lung or visceral pleura, and without evidence of invasion proximal to a lobar bronchus at bronchoscopy.
- T2** A tumour more than 3.0 cm in greatest dimension, or a tumour of any size that either invades the visceral pleura or has associated atelectasis or obstructive pneumonitis extending to the hilar region. At bronchoscopy the proximal extent of demonstrable tumour must be within a lobar bronchus or at least 2.0 cm distal to the carina. Any associated atelectasis or obstructive pneumonitis must involve less than an entire lung.
- T3** A tumour of any size with direct extension into the chest wall (including superior sulcus tumours), diaphragm, or the mediastinal pleura or pericardium without involving the heart, great vessels, trachea, oesophagus or vertebral body, or a tumour in the main bronchus within 2.0 cm of the carina without involving the carina.
- T4** A tumour of any size with invasion of the mediastinum or involving heart, great vessels, trachea, oesophagus, vertebral body or carina or presence of malignant pleural effusion

NODAL INVOLVEMENT (N)

- N0** no demonstrable metastasis to regional lymph nodes.
- N1** Metastasis to lymph nodes in the peribronchial or the ipsilateral hilar region, or both, including direct extension.

- N2 Metastasis to ipsilateral mediastinal lymph nodes and subcarinal lymph nodes
- N3 Metastasis to contralateral mediastinal lymph nodes, contralateral hilar lymph nodes, ipsilateral or contralateral scalene or supraclavicular lymph nodes

DISTANT METASTASIS (M)

- M0 No (known) distant metastasis
- M1 Distant metastasis present—specify site(s)

SUMMARY OF STAGING

Stage 0	TIS		
Stage I	T1	N0	M0
	T2	N0	M0
Stage II	T1	N1	M0
	T2	N1	M0
Stage IIIa	T3	N0	M0
	T3	N1	M0
	T1-3	N2	M0
Stage IIIb	any T	N3	M0
	T4	any N	M0
Stage IV	any T	any N	M1

APPENDIX C

Table describing the apoptotic index, Bcl2 index, CD40 index and other clinico-pathological parameters of the archival series of squamous lung cancers that were investigated in this project.

Case No	Age	Sex	A.I	Bcl2	CD40	Stage	Grade	AgNOR	Ploidy	Survival
110	69	M	33.1	0	0	II	-	-	-	7*
239	50	M	37.4	18.7	0	I	P	14.9	A	21
631	56	M	39.7	100	0	II	M	9.3	A	93*
681	49	F	65.7	100	0	I	P	7.9	A	6
733	70	M	39.1	0	0	I	M	13.4	A	16d
833	57	M	5.7	0	0	III	M	13.9	-	7
1026	52	M	73	0	65.5	IIIa	P	24.0	-	1d
1314	58	M	29.7	65.7	0	I	M	10.7	D	33
1422	60	M	108.7	89.6	100	IIIa	M	15.1	A	3
1602	68	M	12.1	0	0	II	P	12.1	D	19
1603	58	M	23.4	3.6	0	IIIb	P	14.9	A	16
1657	65	F	5.1	0	0	II	M	9.7	D	12
1708	72	M	34.2	66.9	0	IIIb	-	-	-	7
1788	57	F	65.3	12.2	9.5	II	-	-	-	5
1811	54	M	107.2	16.3	100	II	M	12.6	D	15
1808	67	M	87.7	100	100	I	-	-	-	77
1869	63	M	48.9	0	1.2	I	M	10.9	A	17
1967	60	M	145.7	0	0	IIIb	-	-	-	17
2004	62	M	24.7	20.1	0	II	P	22.2	A	29
2016	63	M	20.8	0	26.5	I	W	11.6	D	29
2155	53	M	14.4	0	85.1	IIIb	M	-	D	9
2164	52	M	56.8	0	85.1	I	M	7.5	A	120
2208	57	M	30.8	0	0	IIIb	W	9.7	D	135
2272	68	M	77	0	8.8	I	M	9.9	D	7
2362	66	F	16.5	16.2	7.6	I	P	7.9	A	16
2368	65	M	11.8	94.0	100	II	M	10.4	D	10
2396	62	M	21.8	0	100	I	W	13.3	D	15
2434	63	M	17.3	90.1	0	I	M	11.1	-	118*
2578	58	M	11.7	0	0	I	M	9.7	A	32
2911	57	M	18.5	85.8	0	II	M	9.7	A	25
3065	66	M	11.6	0	13.3	II	M	-	D	32
3098	71	M	48.5	0	0	I	M	14.6	A	29*
3127	53	M	90.8	0	0	II	P	10.3	A	4d
3160	72	M	10.5	2.0	4.1	I	M	6.8	-	5
3176	57	M	19.0	0	97	II	W	7.1	D	133
3434	57	M	19.3	74.6	0	IIIa	-	-	-	72
3478	62	M	88.4	100	0	IIIa	M	-	D	2
3517	40	M	27.4	88.3	0	I	P	13.4	D	4
3686	64	M	16.6	100	75.3	II	P	19.4	-	128
3688	69	M	13.7	0	0	I	P	13.7	A	1d
3709	66	M	14.4	0	0	IIIa	M	11.0	A	28d
3737	63	M	49.9	0	2.1	IIIb	M	13.1	A	8
3744	50	F	46.5	0	100	I	M	5.9	D	10
3896	65	M	49.2	0	100	IIIb	W	9.9	A	5
3884	48	M	48.7	100	12.1	IIIa	M	11.0	D	15
3932	66	M	-	-	-	II	M	4.6	D	17
3996	50	M	49	72.7	0	I	P	16.6	D	132
4141	57	M	22.4	75.2	6.2	II	P	11.4	A	125
4152	61	F	9.2	100	69.2	I	M	7.3	A	72
4158	70	M	12.0	0	0	II	M	12.2	A	23
4196	56	M	52.9	100	88.7	II	P	20.9	A	5
4317	64	M	27.6	10.7	79.1	II	P	-	D	43
4359	67	M	84	0	16.4	IIIa	P	20.5	-	15d
4428	69	M	36.7.0	1.4	88.1	IIIa	P	11.9	A	1d

Case No	Age	Sex	A.I.	Bcl2	CD40	Stage	Grade	AgNOR	Ploidy	Survival
4599	64	M	22.4	96.7	0	I	P	13.1	A	5
4420	54	M	6.4	81.5	8.5	II	M	-	D	7
4728	66	M	48.9	100	0	II	M	6.3	A	46
4784	61	M	75.8	85.8	0	IIIa	W	10.3	A	65
4790	71	M	12	29.2	0	II	P	17.2	D	14
4851	57	M	52.4	0	0	IIIa	W	5.9	D	10
4852	53	F	10.1	0	0	I	-	-	A	131
5048	59	M	16.9	0	100	II	M	11.2	A	29
5057	66	M	31.7	85.2	18.6	I	M	15.9	A	122
5126	64	M	34.1	20.3	0	I	-	-	-	25*
5174	62	M	14.9	0	0	II	M	12.5	A	15*
5265	62	M	6.5	100	0	I	W	15.0	D	129
5327	54	M	14.7	0	0	II	M	9.0	A	10
5461	69	M	10.6	0	0	IIIa	W	13.7	A	24
5521	65	F	8.6	0	100	II	P	8.4	A	22
5672	69	M	-	-	-	I	M	13.0	D	110
5607	71	M	20.8	19.2	33.3	I	M	6.4	A	52*
5620	67	M	22	0	0	II	P	10.5	A	9
5665	65	M	24	52.9	0	I	M	5.9	A	129
5984	65	M	18.3	25.8	59	II	M	18.4	D	6
5986	66	M	60.2	16.8	0	I	-	-	-	33
6057	51	M	31	92.6	0	I	M	9.8	A	54
6180	65	M	52.6	0	0	I	-	-	-	79*
6279	55	M	4.9	0	94.6	I	-	-	-	129
6309	60	M	3.1	3.4	36.8	IIIa	M	10.8	D	17
6318	59	M	4.2	10.5	0	II	P	9.2	D	13*
6372	68	M	7.5	5.2	0	II	-	-	-	13
6486	60	M	47.5	98	0	I	P	11.6	A	23
6517	62	F	38.4	57.5	19	IIIa	M	8.6	A	13
6526	52	M	7.9	0	18.7	I	-	-	D	42*
6572	52	M	31.6	0	0	II	-	-	-	138
6590	73	M	21.3	8	0	I	M	12.2	A	5
6672	61	M	14.1	0	10.3	II	M	11.1	A	10
6676	60	M	16.9	0	93.7	I	-	-	-	42*
6679	58	M	3.6	0	100	IIIb	M	9.2	D	15
6732	65	M	22.4	0	5.1	II	M	5.9	A	7
6792	68	M	23.6	16.3	14.1	II	P	9.8	D	5
6880	54	M	35.7	6.9	0	I	M	13.6	A	14
6879	55	M	10.6	0	7.4	II	-	-	-	18d
6903	60	F	19.8	0	39	II	M	13.6	A	12d
7044	72	M	22.7	100	0	IIIa	P	6.7	-	134
7092	55	M	19.7	0	31.3	I	M	17.2	D	19
7131	57	M	9.9	2.5	0	II	W	8.8	D	35*
7171	66	M	27.7	0	80	II	P	9.1	D	16
7172	68	M	25.6	100	20.4	I	P	14.1	A	133
7259	57	M	4.7	0	0	IV	P	-	D	15
7266	52	M	20.8	7.9	100	II	P	-	D	128
7273	65	M	18.3	100	0	IV	P	10.9	A	10d
7365	56	M	18.6	100	0	II	M	8.1	A	129
7480	64	M	8.3	42	0	IIIa	P	6.6	A	6
7484	68	M	16.0	67	0	II	P	10.1	A	11
7560	62	M	42.8	4.2	3.3	IIIa	P	12.4	A	18
7604	53	F	22	12	0	IIIa	M	10.5	D	35
7700	60	M	10.0	0	0	IIIa	W	13.4	A	28d
7887	67	F	2.6	100	0	IIIb	P	8.1	A	94
7963	64	M	21.7	0	0	IIIa	M	8.4	D	132
8000	43	M	22.7	0	90.3	IIIa	M	9.3	D	4
8157	49	M	29.6	3.3	0	IV	P	8.4	A	1d
7923	63	M	11.4	4.8	0	II	P	-	D	120
8360	62	M	3.9	11.6	0	II	P	10.0	A	21
8395	68	M	24.6	100	0	I	W	8.4	A	25d
8405	57	M	51.1	0	100	IIIa	M	9.8	D	9
8555	64	F	15.7	0	0	IIIa	P	12.9	D	7d
8626	44	M	24.4	77.9	0	I	M	12.9	D	24

Case No	Age	Sex	A.I.	Bcl2	CD40	Stage	Grade	AgNOR	Ploidy	Survival
8711	53	M	23.6	80.4	0	I	-	-	-	132
8741	58	M	16.2	0	0	IIIa	-	-	-	12
8763	60	M	41.2	76.5	0	I	-	-	-	133
8779	58	M	8.1	3.3	78.4	II	M	11.9	A	4
8815	57	M	3.8	0	0	II	M	18.9	A	16
8919	53	M	10.8	0	55.7	II	P	-	D	12
8897	70	M	46.9	80.3	0	II	M	11.0	A	126
8931	62	M	66.2	57.5	8.7	II	P	14.8	D	131
8941	63	M	28.1	0	0	I	M	9.0	A	23
8987	51	M	50.2	0	0	II	P	-	A	8
9073	57	M	13.8	7.5	0	IIIa	M	8.5	D	7d
9074	68	M	69.7	57.9	0	IV	P	5.5	-	6d
9269	65	M	10.9	0	100	I	P	7.4	-	3d
9309	57	M	30.9	88.5	0	IIIa	M	10.1	A	14
9332	57	F	29.2	0	0	I	P	23.6	D	126
9335	57	M	3.9	9.7	9.1	IIIa	M	5.0	A	15
9336	54	M	59.2	19.1	0	IIIb	-	-	-	17
9419	66	M	15.8	10	0	IIIa	M	14.5	A	6
9470	59	M	-	-	-	I	W	9.2	D	49

Key to the table

Case No Serial number of histological slide (year = 1977) from the Pathology Department, East Birmingham Hospital.

Age In years

A.I. Apoptotic index = apoptotic cells/10,000 malignant cells.

Bcl2 Bcl2 index = percentage of Bcl2 positive tumour cells.

CD40 CD40 index = percentage of CD40 positive tumour cells.

Stage See Appendix 2 for staging system employed

Grade Histological differentiation
 W = well differentiated
 M = moderately differentiated
 P = poorly differentiated

AgNOR Mean AgNOR count per cell nucleus per specimen

Ploidy A = aneuploid
 D = diploid

Survival In months except where followed by 'd' = survival in days
 bold = death in immediate post-operative period
 • = death from causes other than lung cancer

"-" No data available

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